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**PROLINE CONCENTRATION AS AN INDICATOR  
OF THE LEVEL OF SALT TOLERANCE**

**Philoména, Y. Rosalie**

**Bachelor of Science (Biological  
Science) with Honours  
1995**

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# **PROLINE CONCENTRATION AS AN INDICATOR OF THE LEVEL OF SALT TOLERANCE**

**Philoména, Y. Rosalie**

Thesis submitted in partial fulfillment of the  
requirements for the award of Bachelor of  
Science (Biological Science) with honours.

Department of Applied Science

Edith Cowan University

November, 1995.

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## ABSTRACT

Each year approximately 20 million hectares of land become affected by increasing salinity. Salt tolerant plants are being used to rehabilitate salt affected areas. Plants use a variety of mechanisms to adapt to salt in their environments. Glycophytes tolerate low to moderate levels of salt while halophytes can tolerate very high salt levels. Many basic physiological attributes have been suggested as important components of a salt tolerant phenotype. These include, influx and/or efflux of ions across plasma membrane and the tonoplast, modification of membrane composition and synthesis of compatible solutes such as soluble carbohydrates, glycine betaines and proline.

The project aimed to determine if proline could be used as an indicator for salt tolerance. To do this, experiments were set up in the glasshouse and in tissue culture to investigate the response to salt of whole plant, plant tissues and cells. The accumulation of proline, fresh weight, dry weight and water content were determined for *A. nummularia*, *A. stipitata* and *F. irregularis*. Shoot cultures of two clones of *A. nummularia* (ANU 001 and 101), *A. stipitata* (AST 001 and 002) and *F. irregularis* (FAN 001 and 102) and three clones of *A. nummularia* (ANU 001, 301 and 302) callus were grown on media containing different levels of salt in vitro. Three clones of *A. nummularia*



(ANU 001, 301 and 302) and *F. irregularis* (FRAN 001, 102 and 203) were grown various levels of salt in a glasshouse.

The proline concentration and productivity was different for all species' shoot cultures and there were differences between clones of *A. nummularia* and *F. irregularis*. The proline concentration of callus cultures increased with increasing salt, while the fresh weight decreased but there was no difference between clones.

In the glasshouse, the clones of *A. nummularia* were not significantly different in proline concentration but accumulated high proline at control and high levels of salt. *F. irregularis* clones responded in a similar way. Further investigation is required to determine how this may reflect variation in salt tolerance.

## **DECLARATION**

**I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.**

**Signature**

**Date: 24 November, 1995**

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## **1. INTRODUCTION**

### **1.1. Salt in the environment**

Approximately ten per cent of the world's land surface is estimated to be adversely affected by salinity. In addition, Malcom (1993) has estimated that 20 million hectares of land deteriorate to a non-useable condition each year. Soil salinity is clearly a major environmental problem. It is mainly caused by clearing of natural vegetation and the subsequent rise in the water table. Geographic distribution of human populations and agricultural practices also contribute to the problem (Flowers *et al.*, 1977).

### **1.2. Salt tolerance in plants**

Plants have different ways of adapting to high salt concentrations in the soil. Salt in the soil causes a change in the osmotic pressure of the external environment, which in turn affects the osmotic potential inside the plant (Flowers *et al.*, 1977; Greenway & Munns, 1980). The effects of high external ion concentration can result in the reduction in turgor, inhibition of enzyme activity, inhibition of photosynthesis, increased use of metabolic energy for non-growth processes, inhibition of membrane function or an inability to eliminate ions due to inadequate transport (Flowers *et al.*, 1977; Greenway and Munns, 1980; Briens and Larher, 1992).



Generally plants can be identified on the basis of their response to salt in the environment. Halophytes are those plants that are able to grow in the presence of high salt concentrations while glycophytes are plants that are sensitive to high levels of salt, but can tolerate moderate levels (Salisbury and Ross, 1992). Halophytes and glycophytes have different responses to salinity. Halophytes respond to high salinity by tolerating high ion content while glycophytes respond to salinity mainly by ion exclusion (Flowers *et al.*, 1977).

The majority of glycophyte species are leaf excluders and may accumulate high levels of  $\text{Na}^+$  in their roots and stems. The exclusion of  $\text{Na}^+$  in the shoots results in little growth increase when glycophytes are subjected to saline environments. The reduction in growth is also a result of a decrease in cell enlargement due to the inability of the cells to maintain turgor. Glycophytes adapt to moderate salt stress after the initial decrease in turgor.

Halophytes are the only plants that can accumulate concentrations of salt at or above that of sea water. Sodium chloride is the most common source of salinity although halophytes are able to accumulate various other ions to very high levels. On average, more than 90% of the  $\text{Na}^+$  in halophytes is in the shoots and at least 80% in the leaves (Flowers *et al.*, 1977). Casas *et al.* (1991) found that there was an initial decrease in growth in *Atriplex nummularia* when subjected to a moderate level of salt in tissue culture. They reported that the decrease in growth was not caused by reduced turgor, as in the case of glycophytes, but as a result of reduced cell division. Normally the expansion rate of halophyte cells appear to be slow relative to that of glycophytes. It

was suggested by Casas *et al.* (1991) that this could be due to a mechanism that contributes to the maintenance of water status and regulation of ion pools.

Turgor is maintained in halophytes by compartmentalisation of ions into the vacuole and neutral organic solutes in the cytoplasm. These halophytes avoid excess internal ion concentration by controlling the uptake of soil solution that is transported to the shoot. Salt is also taken from the shoots by salt glands and bladders. The shoots of these plants are usually succulent. When there is an excess of ions in the cytoplasm and cell wall, water deficit develops in the protoplast of the cell and there is a decrease in carbon dioxide fixation (Greenway & Munns, 1980; Hasegawa *et al.*, 1986; Flowers *et al.*, 1977).

### **1.3. Mechanism of salt tolerance**

A number of mechanisms are utilised by plants to adapt to salt in their environment. These mechanisms have been intensively researched and as a result the physiology of plants under salt stress has been greatly understood. This has also provided reference for the production of more salt-tolerant genotypes through breeding plants that have shown resistance to high salt environments.

The mechanisms of salt tolerance in plants include both physiological and physical changes and adaptations. Glycophytes have roots that have a second layer of endodermis, which helps in the ion-exclusion process. The parenchyma cells of the root xylem extract salts from the solution going to the shoot. Other features of

glycophytes include the exchange of materials between the xylem and the phloem and distribution of ions between the growing and non-growing regions of the plant (Wyn-Jones and Gorham, 1983).

In most halophytes the shoots have salt-glands or bladder cells that extract salts. *Atriplex halimus* has leaves that are covered with several layers of hairs. Mozafar and Goodin (1970) found that the concentration of  $\text{Na}^+$  and  $\text{K}^+$  ions were very high in these balloon-like cells, and the concentration increased in plants growing on saline media. They found that osmotic adjustment was absent in *A. halimus* and concluded that because the vesiculated hairs increased in salt concentration as a result of salinity, then they were used as a means of removing salt from the leaves.

Halophytes also maintain turgor in the cells by synthesising organic solutes. Their roots tend to be more permeable to water and the leaves are thicker than those of glycophytes. In the instance of water deficiency, there is a decrease in growth in halophytes due to a decrease in carbon dioxide fixation (Flowers *et al.*, 1977; Greenway and Munns, 1980; Hasegawa *et al.*, 1986). According to Greenway and Munns (1980) it is difficult to say if the reduction in growth in glycophytes is due to water stress or ion excess and in what tissue the primary effect is felt.

Various studies have shown that the accumulation of organic solutes such as organic acids, nitrogen compounds and carbohydrates contribute to the osmotic adjustment of the cytoplasm (Flowers *et al.*, 1977). Most plants studied have been found to accumulate a range of organic compounds when subjected to salinity.

*Mesembryanthemum crystallinum* (ice plant) switches from C3 to crassulacean acid metabolism (CAM). As a result of the switch, *M. crystallinum* accumulates malate when grown on a saline medium (Flowers *et al.*, 1977; Ostrem *et al.*, 1987; 1990). Hellebust (1976) gave evidence that there is a linear increase of the organic acid mannitol, in *Platymonas suecica* with increasing external salinity.

One nitrogen compound of great interest that accumulates in plants when exposed to salt stress is proline. Proline is found in all plants as free proline or attached to proteins. Several studies using <sup>14</sup>C- labelled precursors, have indicated that glutamate is a major precursor of proline in plants that are subjected to salt stress. The present evidence suggests that the increase in the rate of proline synthesis may partly involve the activation of enzymes of proline biosynthesis, possibly coupled with a decrease of proline feedback inhibition of the pathway (Bogess *et al.*, 1976; Delauney and Verma, 1993; Stewart, 1981; Fig. 1).

Decreased proline oxidation to glutamate, decreased utilisation of proline in protein synthesis and enhanced proline production may all contribute to net proline accumulation. There are indications that transcription and translation are required for proline biosynthesis in plants. Ornithine has also been identified as a source of proline (Samaras *et al.*, 1995).



proline can function as a hydroxy radical acceptor and may stabilise membranes by interacting with phospholipids. Proline is also compatible with cell metabolism and can be converted to glutamate, which plays an important role in the synthesis of essential amino-acids (Ashraf, 1994).

Although there is a significant number of studies on the level of proline in salt tolerant plants, there has been few that examine the differences in levels between different species, and different clones or varieties of a species. Daines and Gould (1985) found that the final amount of proline in tissue cultures of the halophytic grass *Distichlis spicata*, was positively correlated with the level of salt stress. They concluded that the level of proline is an indicator of salt tolerance. The study did not, however, determine the level of proline increase in different species or in different clones of the same species. Demmig and Winter (1986) produced similar results in their work with *M. crystallinum*, but again the study used only one clone. Ashraf (1994) investigated salt tolerance in three clones of the pigeon pea (*Cajanus cajan*). He found that there was a genetic variation for salt-tolerance among the three clones and that proline and carbohydrate accumulation was highest in the more salt-tolerant clone

#### **1.4. Tissue culture and breeding for salt tolerance**

Breeding for salt tolerance has been difficult in the past because of various difficulties relating to techniques and general physiology of plants. There are various criteria that have to be considered when improving salt tolerance in plants. These include the

ability of the tolerance-improved plants to grow vigorously in saline conditions, suitable genetic variability in the parent species and other related plants, a suitable method for screening large numbers of clones for salt tolerance and an analytical method to identify salt-tolerant clones in large populations (Tal, 1985). The last criterion is one of the major problems that faces studies in breeding for salt-tolerance, while extensive research is being done to develop and improve other factors that affect breeding experiments, such as selection for suitable genetic variability and screening clones for salt-tolerance.

Various properties and parts of plants have been considered essential for growth in saline conditions. These provide targets for research in plant tolerance to stress. The targets include, growing regions, energy related physiological processes, membrane functions, osmoregulation, role of macromolecules, hormones and essential nutrients (Tal, 1989).

In 1878, Vochting, a german botanist, stated that every plant fragment, however small, contains the elements that can build up the whole plant. This was after he attempted to investigate the factors which play a role in the control of organ formation and differentiation in plants. This was considered the birth of the ideas of tissue culture (Mantell *et al.*, 1985).

Tissue culture has now been proven to be a successful technique for selecting plants for a number of adaptations. Intensive research has been done to improve the technique and in doing so various findings on the relationship between *in vitro* and *in vivo* propagation have been published. Micropropagation allows the rapid production

of a large number of plants as well as screening for physiological properties and diseases that affect plants (Mantell *et al.*, 1985)

In breeding, micropropagation is useful for the maintenance and multiplication of a suitable number of genotypes or potential new cultivars including any products of genetic engineering involving *in vitro* procedures. At present micropropagation is used for the production of pathogen-free plants, germplasm storage, seed production and mass propagation of new genotypes. Germplasm storage provides genetic material from ancient plant varieties and their wild relatives. With increasing land-clearing activities, natural germplasm is being lost on a large scale and this could be a setback for future breeding programmes. Mass propagation is fast replacing seed production from cloned parents. While seed production is very important in the agricultural industry where the growth of the plant can be monitored, mass propagation is used in rehabilitation and revegetation of degraded land. Mass production is considered more advantageous than seed production because of the high degree of genetic conservation that is provided in the latter. The former also allows the propagation of plants which would otherwise only be propagated by seed. This is particularly true for crop palms such as coconut, oil and date palm (Mantell *et al.*, 1985).

In breeding for salt tolerance, a useful technique is the production of mutants of wild type plants through tissue culture methods. The mutation may be permanent and thus remain stable through consecutive cell generations. Saleki *et al.* (1993) found that mutant strains of *Arabidopsis thaliana* showed the ability to germinate in more saline conditions than the wild type. Tissue culture studies have also indicated the upper



limit of salt tolerance between species of the same genus. It has been found that the upper limit for halophytes (e.g. *Atriplex vesicaria* and *A. hastata*) in tissue culture is in excess of 300mM NaCl (Flowers *et al.*, 1977).

The plants regenerated from tissues culture that have been selected for salt tolerance have shown variable responses to salt at the whole plant level. McCoy (1987) evaluated the relationship between tolerance of different genotypes *in vitro* and *in vivo* in several *Medicago* species. It was found that all the genotypes that showed tolerance to NaCl *in vitro* did not show tolerance *in vivo*. It was also found that *Medicago marina* had the most NaCl sensitive genotypes at the *in vitro* level, but exhibited NaCl tolerance at the whole plant level. Johnson and Smith (1992) found that there was an increase in tolerance of plants derived from callus cultures grown on saline media in alfalfa. This was in agreement of the work done by Smith and McComb (1981; 1983) in their work with *Atriplex undulata*, *Suaeda australis* and *Medicago sativa*.

### 1.5. Uses of halophytic plants

Salt tolerant plants have been used for a number of purposes including, rehabilitation of salt affected areas and as forage for livestock in arid and semi-arid areas. In Pakistan, India and Australia, the revegetation of salt-affected areas with halophytes is well established (Malcom, 1993; Hanjra and Rasool, 1993; Quereshi *et al.*, 1993; Rashid *et al.*, 1993).

In Western Australia useful grazing is obtained from natural stands and from introduced salt tolerant plants. These plants are invaluable because they are available during adverse conditions, such as drought (Clarke, 1982).

Two Western Australian halophytes, *Atriplex* and *Frankenia*, are considered to have potential for the rehabilitation of salt-affected areas. *Atriplex* is distributed throughout the world and is important in rehabilitation programs in salt-affected regions and areas where the vegetation has been cleared for agricultural purposes. *Frankenia* is a less known and less researched genus and there is little published information about its present uses.

The family Chenopodiaceae contains more than 100 genera and 1, 500 species, many of which are glycophytes. The genus *Atriplex* is particularly important within the family having over 250 species. This is a cosmopolitan genus, with 60 Australian native species and several introduced species (Wilson, 1984).

Many species of *Atriplex*, such as *A. vesicaria* (bladder saltbush), are considered invaluable fodder for stock during long, dry periods while other species are consumed by humans. *A. cinerea* was once used as a pot-herb in New South Wales. Navajo Indians of North America ground the seeds of *A. canescens* (four-winged saltbush) into a flour and used it for porridge. They also chewed the stems and applied the pulpy mash to the swelling caused by insect bites. *A. nitens* was used for centuries in Europe as a vegetable for stews and soups. *A. hamiloides*, has been used as a treatment for jaundice (Encyclopaedia Botanica, 1988; Cribb and Cribb, 1981).

*Atriplex nummularia* (old man saltbush) is a species that has been divided into several subspecies which are distributed throughout inland Australia. It is commonly used as fodder and it has high nutritional value. Sheep that feed on *A. nummularia* are said to remain free of fluke and are cured of Distoma-disease and of other related ailments. Early settlers also used it to treat blood disorders. This species has become scarce in some areas because of overgrazing (Cribb and Cribb, 1981).

The family Frankeniaceae is another cosmopolitan group with 4 genera and about 100 species. Only one genus, *Frankenia*, occurs in Australia (Barnsley, 1982). In Western Australia, they are distributed between the Pilbara and the Nullarbor. They are found in saline areas and the group contains ten per cent salt and is only used as forage if stock water contains very little salt. During drought periods, they provide very useful forage. Frankenias are mostly resistant to grazing and they are good indicator species where the stock water contains less than 2000 ppm of salt. Where the stock water has a higher salt content *Frankenia* has no indicator value (Mitchelle and Wilcox, 1988). Very few uses of the *Frankenia* species have been reported but at present they are considered potentially useful for rehabilitation of salt-affected areas.

## 1.6. Aims

To be able to obtain species that are more tolerant to the ever increasing saline conditions of the soil, it is important to have a fast and reliable technique to test for salt-tolerance. Genetically viable species and varieties can be produced quickly and rehabilitation of salt-affected areas will become more efficient.

This project aimed to use proline to differentiate variation in salt tolerance between clones of three halophytic species, *Atriplex nummularia*, *A. stipitata* and *Frankenia irregularis*. Clones of these plants were produced from cuttings from plants that have survived high saline conditions on mine sites in Kalgoorlie and Norseman. The plants were subjected to various levels of salts in the glasshouse and *in vitro* as shoot and callus cultures.

The results of the project have provided information on the differences between clones in response to salt; differences between tissue types and responses to salt; and the ability to use proline as a measure of these responses.

### **1.7. Hypotheses that were tested**

1. There is genotypic variability in salt tolerance between individuals of the halophytes *Atriplex nummularia*, *A. stipitata* and *Frankenia irregularis*.
2. Differences in productivity can be used to measure the differences in salt tolerance between individuals of the same species.
3. Proline concentration can be used to compare salt tolerance between species.
4. There is genetic variability in proline concentrations between individuals of the same species.
5. Proline concentration can be used to measure genotypic differences within a species in salt tolerance at a cellular (callus), tissue (shoots *in vitro*) and whole plant level.

## **2. GENERAL MATERIALS AND METHODS**

### **2.1. Plant materials**

Plant species used in the project were *Atriplex nummularia* (ANU), *Atriplex stipitata* (AST) and *Frankenia irregularis* (FRAN). *A. nummularia* and *F. irregularis* were used both in the glasshouse and in tissue culture, and *A. Stipitata* was only used in tissue culture experiments. In the glasshouse three clones of *A. nummularia* and *F. irregularis* were used, ANU 001, 301 and 302, Fran 001, 102 and 203. Two clones of *A. stipitata* were used in tissue culture AST 001 and 002. Table 1.1 shows the origins of the different clones.

### **2.2. Tissue culture techniques**

#### **2.2.1. Media preparation**

The basal growth medium for shoots and callus contained Murashige and Skooge (M & S) (1962) minerals, vitamins and inositol (Table 1.2). Media were prepared by mixing 4.43g of M & S (Sigma M5519) powder and 20g sugar to 90 mL ultra pure water. For shoot medium 1  $\mu$ M kinetin was added and for callus medium 9  $\mu$ M naphthalene acetic acid (NAA) and 9  $\mu$ M benzylamino purine (BAP) were added. The mixture was then made up to 1 L, the pH was adjusted to 5.8, and 2.5g phytigel and 2.5g agar added and dissolved by heating. The medium was then dispensed (50 mLs) into 250 mL polycarbonate containers. The containers were then autoclaved for 15 minutes at 121° C. Analytical grade salt was used in media for salt treatments and control levels contain salt NaCl concentration of M & S.

**Table 1.1.** Origins of the different clones used in tissue culture and glasshouse experiments.

Species/ clone	Origin	Selection criteria
<i>A. nummularia</i>		
001	commercial clone	none
301	Chinaman creek,	saline creek
302	Chinaman creek,	saline creek
<i>A. stipitata</i>		
001	Kalgoorlie	saline mine site
002	Kalgoorlie	saline mine site
<i>F. irregularis</i>		
001	Kalgoorlie	saline mine site
102	Kalgoorlie	saline mine site
203	Norseman	saline mine site

### 1.2.2. Sterile Techniques

All instruments used to handle explants and callus were sterilised in the autoclave for 15 minutes at 120° C. All the subculturing was carried out in a laminar flow unit. Before use, the unit was swabbed with 70% ethanol and during subculture instruments were periodically resterilised in a bacticinerator (Sigma S3273) and surgical masks and gloves were worn.

**Table 1.2.** Components of Murashige and Skoog (1962) basal medium

Components	mg/L
Ammonium Nitrate	1650.0
Boric acid	6.2
Calcium Chloride anhydrous	332.2
Cobalt Chloride. 6H <sub>2</sub> O	0.025
Cupric Sulfate. 5 H <sub>2</sub> O	0.025
Na <sub>2</sub> -EDTA	37.26
Ferrous Sulfate. 7H <sub>2</sub> O	27.8
Magnesium Sulfate	180.7
Magnesium Sulfate. H <sub>2</sub> O	16.9
Molybdic acid (Sodium salt). 2H <sub>2</sub> O	0.25
Potassium Iodide	0.83
Potassium Nitrate	1900.0
Potassium Phosphate Monobasic	170.0
Zinc Sulfate. 7H <sub>2</sub> O	8.6
Glycine (free base)	2.0
myo-inositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine. HCl	0.5
Thiamine.HCl	0.1



### **2.2.3. Culture initiation**

#### **Shoot cultures**

Shoot cultures were set up using already established cultures. The shoots were subcultured at four weekly intervals to produce sufficient material for experimentation.

#### **Callus cultures**

Callus cultures were initiated by using leaf tissue from established glasshouse plants. Whole leaves were surface sterilised by shaking in 2% Zephiran in 10% alcohol for 5 minutes. The leaves were then rinsed three times in sterile water. Squares of leaf tissue of approximately 1 cm by 1 cm were cut and placed in 30 mL tubes containing 10 mL callus growth medium. Subculturing was done by cutting the growing calli in halves until enough tissue was obtained for experimentation.

### **2.2.4. Environmental conditions**

Cultures were maintained in a culture room at  $25^{\circ}\text{C} \pm 2$  with a photoperiod of 16 hrs light/ 8 hrs dark. Light intensity was supplied by 4 (36 Watts) bulbs.

## **2.3. Glasshouse techniques**

### **2.3.1. Cutting preparation**

Cuttings of approximately 5 cm were taken from established plants and placed in 1 mM indole-3-butyric acid (IBA) for approximately 30 minutes. The cuttings were then dipped in rooting hormone (Clonex<sup>®</sup>) before being put in a pasteurised mixture of peat, perlite, fine sand and river sand mixed in equal proportions. The plants were kept in a misting cabinet for 4 weeks, and then hardened off in a shade house for 1 week.

### **2.3.2. Soil preparation**

Coarse and fine sand in the ratio of 2:1 were mixed and steam pasteurised for approximately 30 minutes. The mixed soil was dried and 4 kg of the dried soil was placed in pots with a single drainage hole. Where varying concentrations of salt were administered, control levels contained NaCl concentration of Thrive.

## **2.4. Proline test**

All glasshouse plants, tissue culture shoots and callus were harvested and tested for proline. The proline test was developed and performed according to the procedure described by Bates *et al.*, 1973.

Fully expanded leaves from the glasshouse plants were sampled and 0.5 g of *A. nummularia* and 0.1 g of *F. irregularis* were used. For the material from tissue

culture, whole shoots and whole calli were used. The plant samples were homogenised in 10 mL sulfosalicylic acid, the homogenate was centrifuged at high speed for 10 minutes and 2 mL of the supernatant was reacted with 2 mL acid ninhydrin and 2 mL glacial acetic acid. The reaction mixture was incubated at 100°C for 1 hour. The reaction was then terminated in an ice bath. The chromophore was extracted by adding 4 mL toluene and mixing with a stirrer for 15-20 seconds. The absorbance of the chromophore was read on a spectrophotometer at 520 nm using toluene as a blank. The acid ninhydrin was made up by reacting 1.25 g ninhydrin, 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid to make up 50 mL.

The proline concentration was determined from a standard curve and the concentration per gramme was calculated using the following equation.

$$\frac{\mu \text{ mole / mL} \times \text{mL toluene} \times 5}{\text{g sample}} = \mu \text{ mole proline / g fresh weight}$$

## 2.5. Growth measurements

Growth was determined by measuring the fresh weight, dry weight and % water content of the plants. For plants growing in the glasshouse the mass of the shoot and roots were determined separately. In tissue culture all the above measurements were made for shoots and only fresh weight was determined for callus. Dry weight was determined by placing the plants in the oven at 60° C until they were at a constant weight.

## **2.6. Data analysis**

Two-way analysis of variance (ANOVA) was used to test for differences between clones and effects of treatments. Where there was a difference due to treatment one way ANOVA was performed on individual clones to test if there was a difference in proline concentration, fresh weight, dry weight and % water content. Duncan's multiple range test was performed on clones where there was a significant difference between treatments.

### **3. TISSUE CULTURE EXPERIMENTS**

#### **3.1. Introduction**

Tissue culture is a widely used technique for the screening of plant traits and adaptations. It is a fast and inexpensive way for screening plants under controlled conditions. Plants grown in tissue culture have been shown to behave differently towards salt in their environment compared to plants grown in the glasshouse (McCoy, 1987; Johnson and Smith, 1992; Smith and McComb, 1981, 1983).

The effect of varying salt levels on proline accumulation and growth were studied on shoot cultures of *A. nummularia*, *A. stipitata* and *F. irregularis* and callus cultures of *A. nummularia*. Different approaches have been used to study the response of tissues to salt *in vitro*. The type of approach used can cause tissues to respond differently to salt. In this project, the plants were adapted to salt by sequential transfer of explants to media with increasing concentrations of NaCl.

## 3.2. Materials and methods

### 3.2.1. Shoot cultures

Shoots of *A. nummularia*, *A. stipitata* and *F. irregularis* were grown on M & S medium with 1  $\mu$ M kinetin (section 2.2.1) until they were of a useable size and quantity. Shoots were then transferred to media with a range of NaCl concentrations (0 mM 50 mM, 100 mM, 200 mM and 400 mM). The shoots were transferred to these media by subculturing every three days to higher levels of salt. Those that had reached the required salt concentration were also subcultured but onto the same medium. Three tubs of each concentration were set up per clone and there were 5 shoots per tub. An initial experiment was established with a single clone of *A. nummularia* (ANU 001) and *F. irregularis* (FRAN 102). Subsequently, the experiment was repeated using two clones of each species: ANU 001, 102; AST 001, 002; and FRAN 001, 102. Once the required salt concentrations were reached the shoots were maintained for 4 weeks. After the 4 weeks the shoots were harvested and proline concentrations, fresh weight, dry weight and water content measured.

### 3.2.2. Callus cultures.

Callus cultures of *A. nummularia* (ANU 001) were initiated as described in chapter 2 (section 2.2.3) and grown on M & S medium containing 9  $\mu$ M BAP and 9  $\mu$ M NAA until sufficient material was available for experimentation. Pieces of callus approximately 1 cm x 1 cm were transferred onto media containing different salt concentrations (0 mM, 50 mM, 100 mM, 200 mM and 400 mM) using the same approach as for the shoot cultures. After transfer to the desired concentrations the calli were grown for a further 4 weeks. Whole calli were randomly selected for the proline determination (3 calli per treatment) and the fresh weight of all the calli was measured. This experiment was repeated using three clones of *A. nummularia* (ANU 001, 301, 302).

A similar experiment was attempted with three clones of *F. irregularis* but the callus initiation was insufficient to replicate the experiment.

## **3.3. Results**

### 3.3.1 Shoot cultures

#### *A. nummularia*

The preliminary experiment using ANU 001 indicated that for all the parameters measured there was no significant effect of increasing the level of salt in the medium. The one way ANOVA suggested that the level of salt did not affect the

proline production in this clone. However, there was a trend for decreased proline with higher salt, and the productivity also appeared to change with the different salt concentrations (Fig. 3.1 a-c). In addition, it was observed that the plants showed considerable chlorosis at concentrations of 200 and 400 mM NaCl.

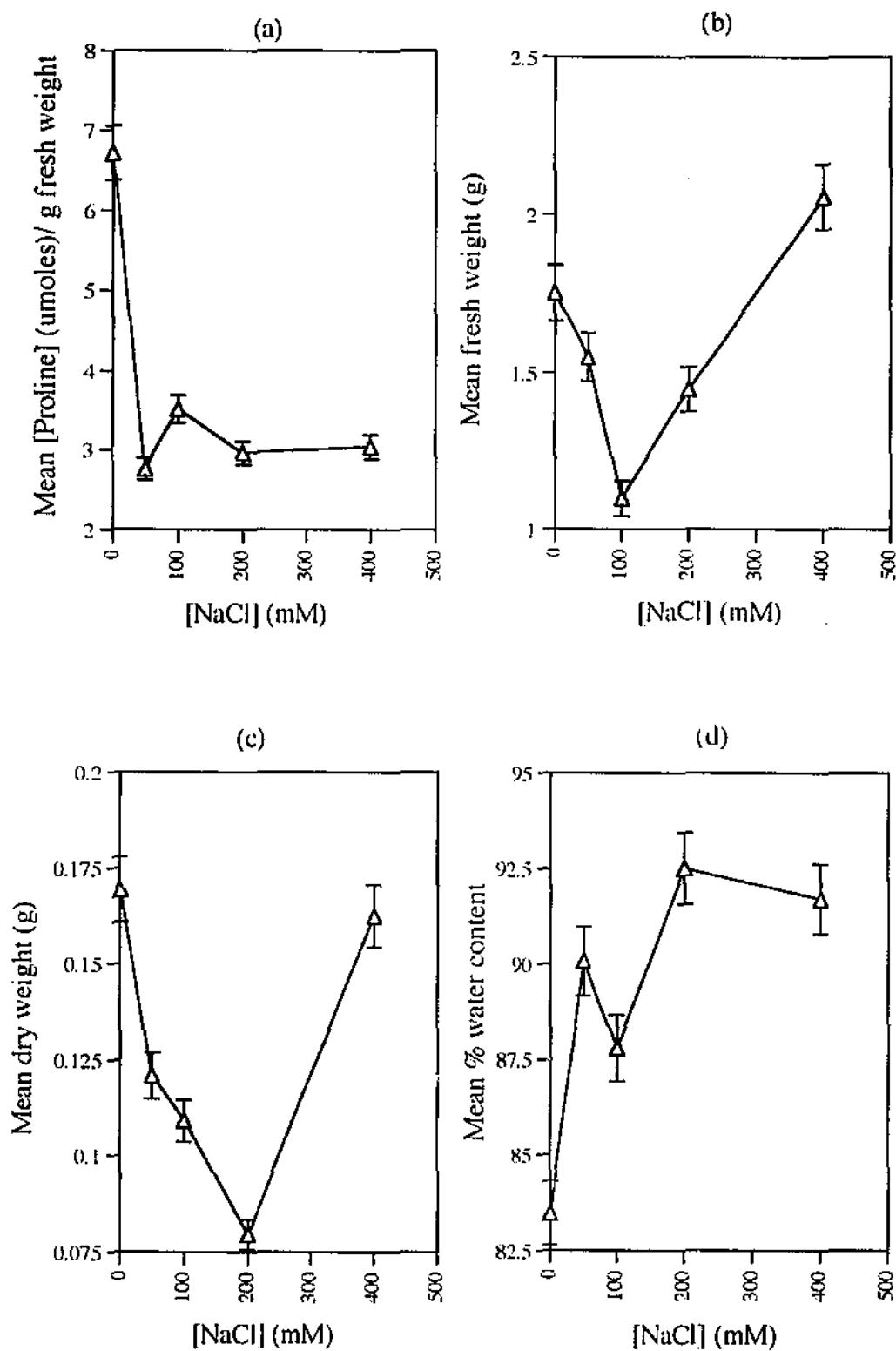
When this experiment was repeated and two clones were used, a different result was obtained. There was a significant difference between the clones in the amount of proline produced and in the fresh weight (Fig. 3.2 a & b). There was also a difference in the way each of the clones responded. For ANU 001, unlike in the initial trial, there was a significant change in the level of proline due to the salt treatments. However, for ANU 101 there was no difference in the proline production (Fig. 3.2a). There was no differences obtained either due to clonal variation or treatment using the dry weight or water content values (Fig. 3.2c & d).

#### *A. stipitata*

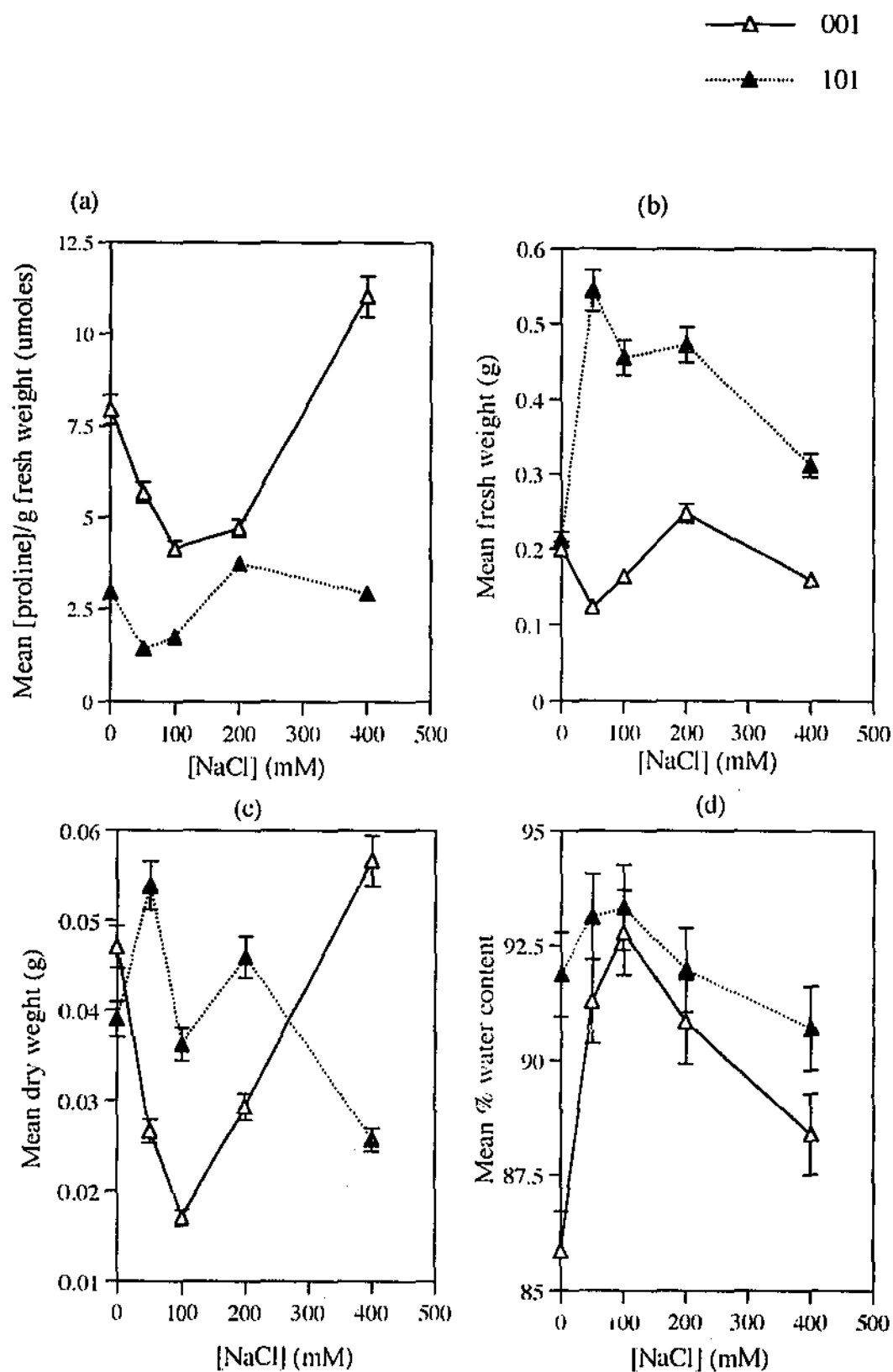
For the two clones of *A. stipitata* there was no difference between the clones for any of the parameters measured (Fig. 3.3). The trend in proline concentration does not match that of any of the other plants investigated. The treatments produced a difference in fresh weight at the different levels of salt (Fig 3.3b) and the growth increase was significant at 200 mM for both clones. This fresh weight



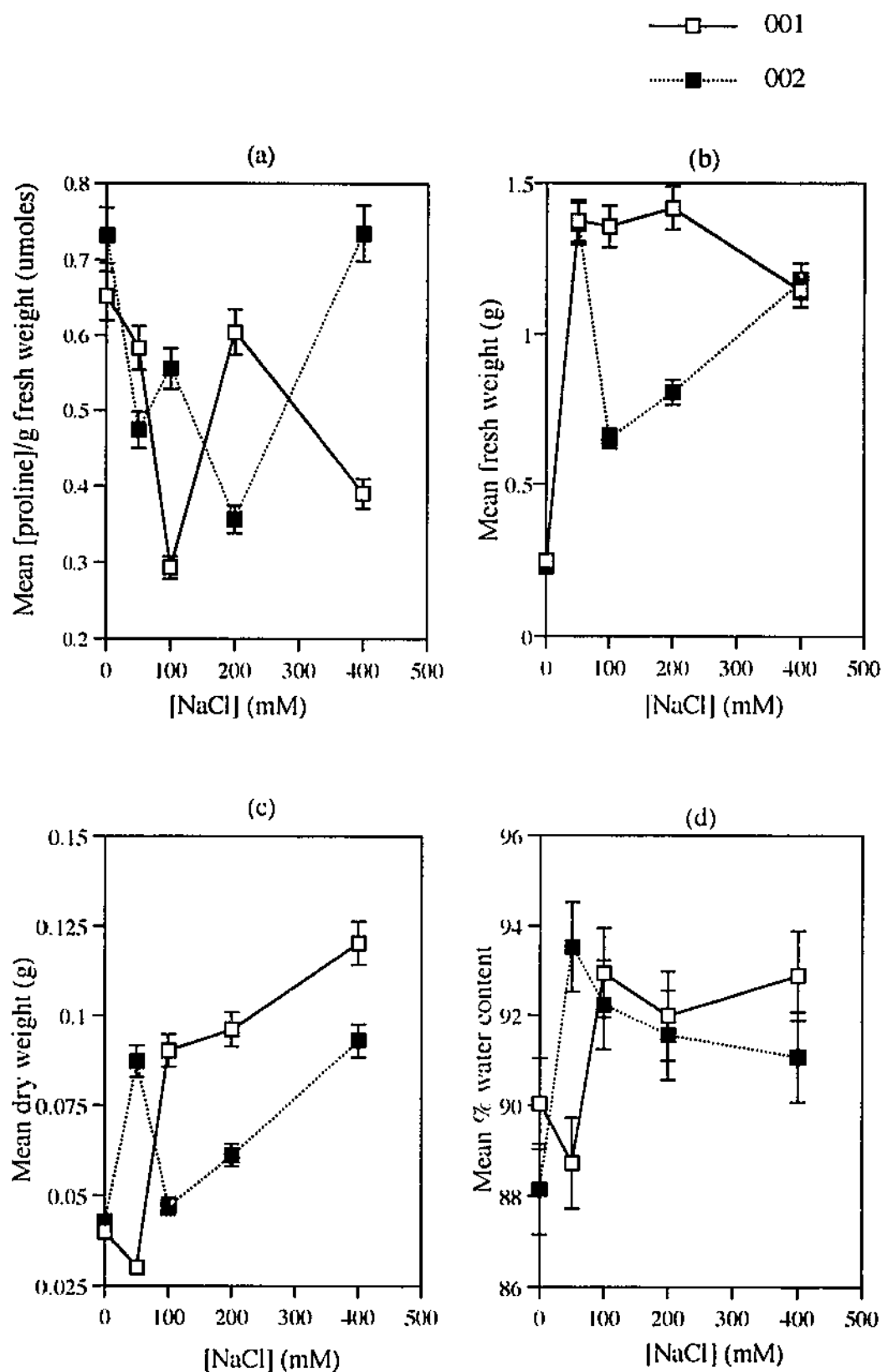
**Fig. 3.1.** Mean proline concentration (umoles/g fresh weight) (a), fresh weight (b), dry weight (c) and % water content (d) of *A. nummularia* shoots (clone 001) grown in tissue culture. Shoots grown on media containing varying NaCl concentrations. Means were calculated from 5 replicates and vertical error bars show standard error.



**Fig. 3.2.** Mean proline concentration (umoles /g fresh weight) (a), fresh weight (b), dry weight (c) and % water content (d) of ANU 001 and 101 shoots grown in tissue culture at varying NaCl concentrations. Means were calculated from 5 replicates and vertical error bars show standard error.



**Fig. 3.3.** Mean proline concentration (umoles/g fresh weight) (a), fresh weight (b), dry weight (c) and % water content (d) of AST 001 and 002 shoots grown in tissue culture at varying NaCl concentrations. Means were calculated from 9 replicates and vertical error bars show standard error.



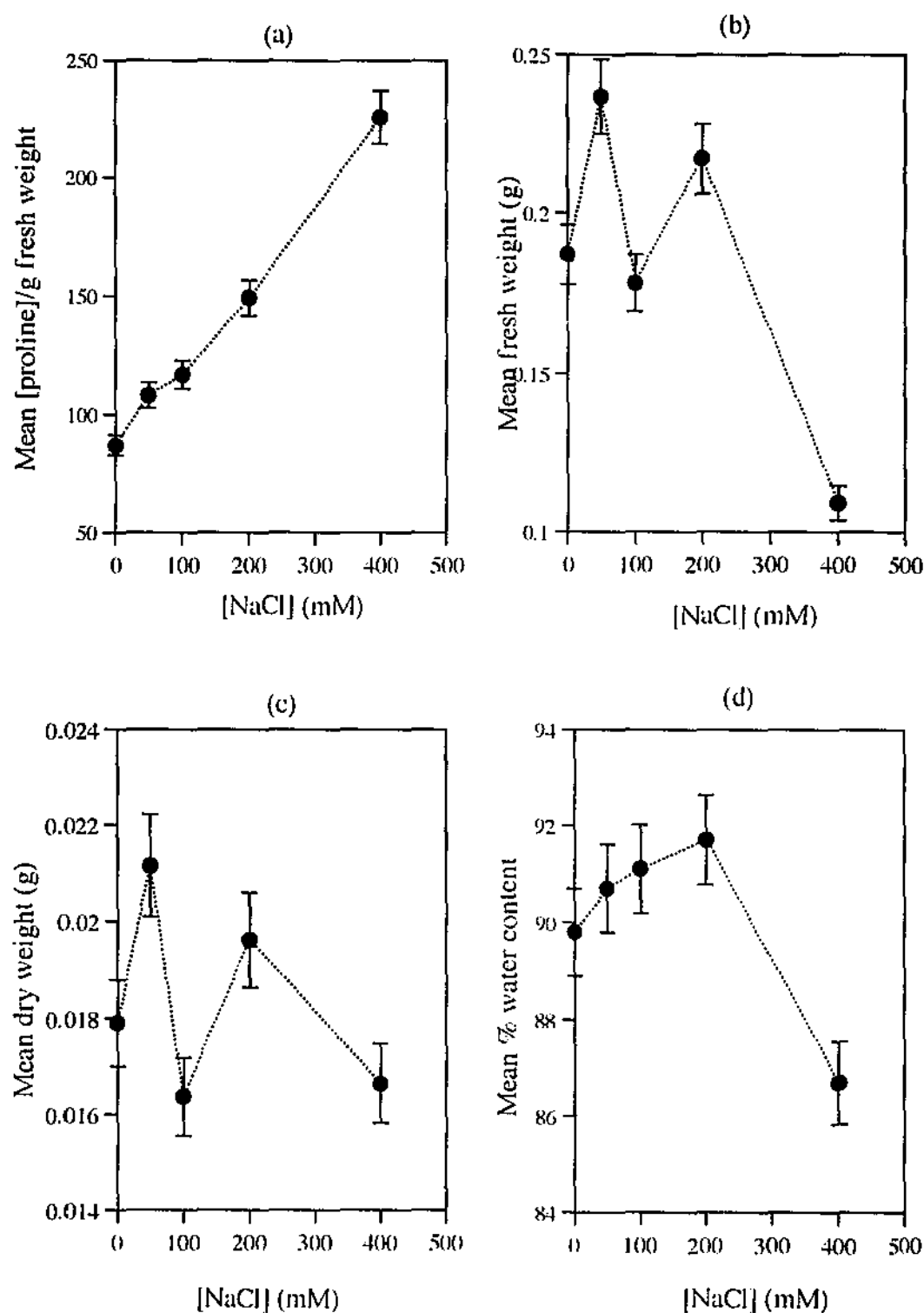
increase is a trend that is also seen in the dry weights but this is not significant (Fig. 3.3c). The percentage water content significantly increased with the increase in the level of salt (Fig. 3.3d), as was seen with *A. nummularia*.

#### *F. irregularis*

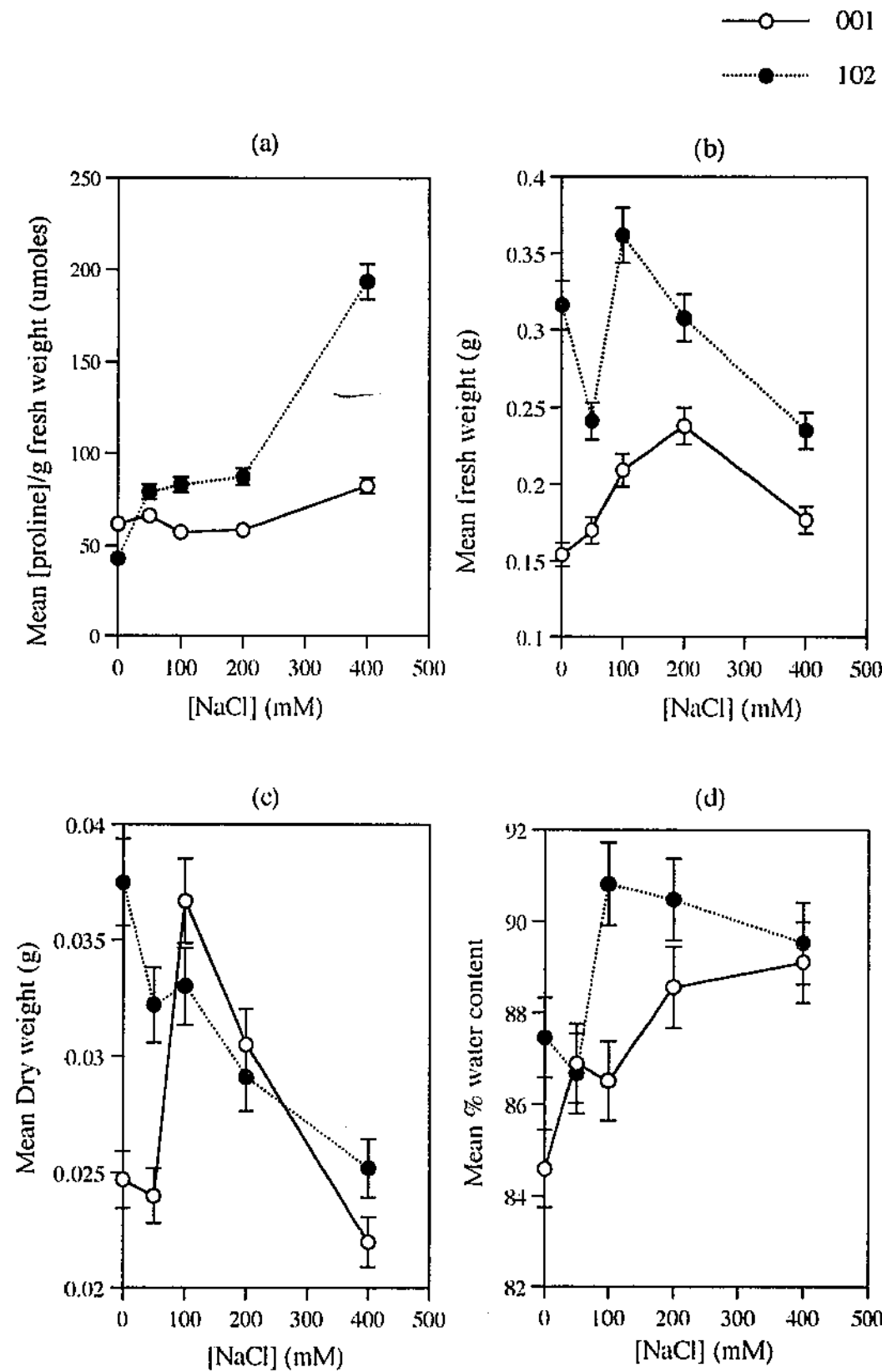
The preliminary experiment with FRAN 102 indicated that proline increased with increasing salt, but there was no significant difference in the other criteria measured (Fig 3.4). The multiple range test indicated that proline accumulation at 400 mM was significantly higher than at the other salt levels.

In the repeat experiment with FRAN 001 and FRAN 102 there was a significant difference between the clones in all the criteria measured (Fig 3.5). The amount of proline increased with increasing salt for FRAN 102 but not for FRAN 001 where there was no effect of treatment (Fig. 3.5a). Similarly, there was no effect of treatment on fresh weight, dry weight or water content for FRAN 001 but a difference due to treatment in water content for FRAN 102 (Fig. 3.5d). For this clone the percentage water content increased with increasing salt concentration, similar to that seen in *Atriplex*. The three higher salt concentrations were significantly different from 0 mM and 50 mM, a trend that was also evident for FRAN 001 (Fig. 3.5d).

**Fig. 3.4.** Mean proline concentration (umoles/g fresh weight) (a), mean fresh weight (b) mean dry weight (c) and mean % water content (d) of *F. irregularis* shoots (clone 102) grown in tissue culture at varying NaCl concentrations. Means were calculated from 5 replicates and vertical error bars show standard error.



**Fig. 3.5.** Mean proline concentration (umoles/g fresh weight) (a), fresh weight (b), dry weight (c) and % water content (d) of FRAN 001 and 102 shoots grown in tissue culture at varying NaCl concentrations. Means were calculated from 9 replicates and vertical error bars show standard error.



### 3.3.2. Callus cultures

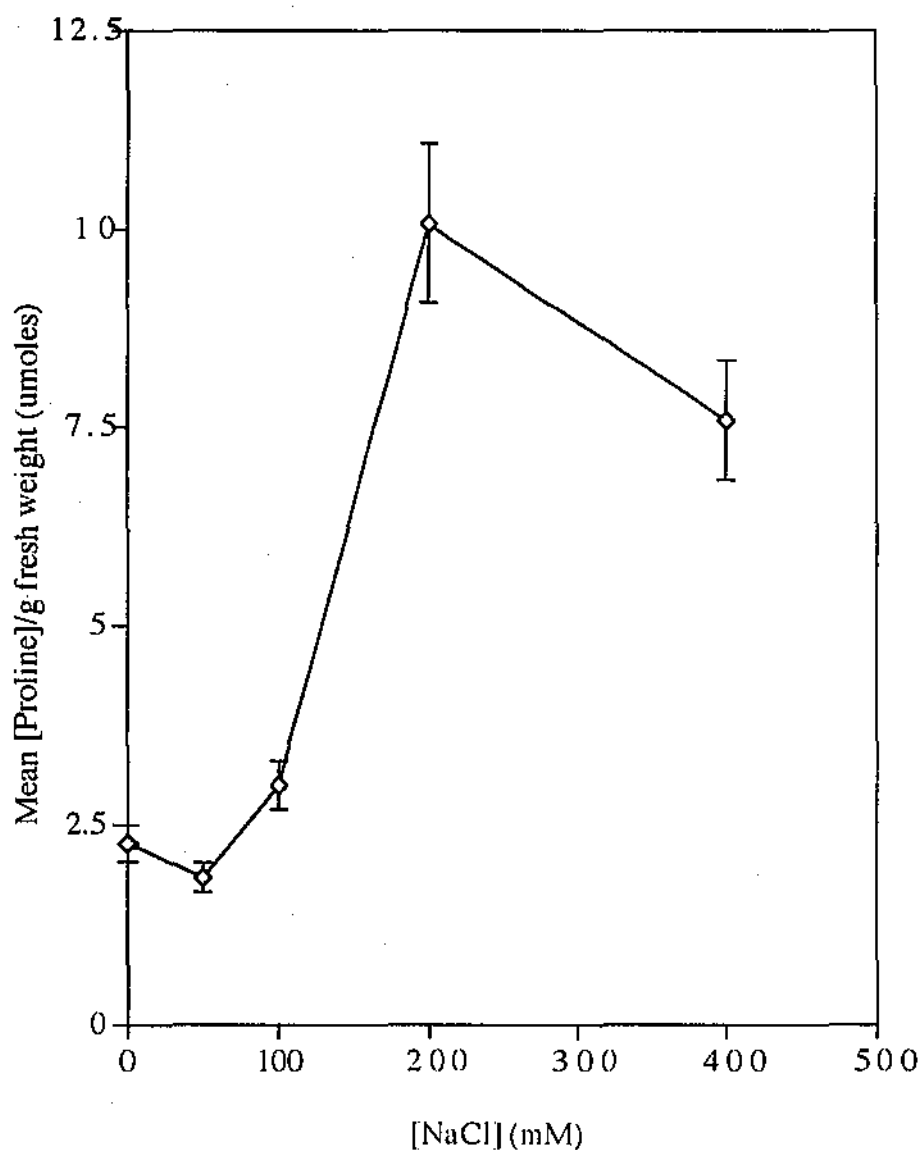
There were distinct differences in the appearance of the calli of the three clones of *A. nummularia*. Some ANU 301 and all ANU 302 calli were brown in colour and had a friable texture. ANU 001 calli were green in appearance and non-friable.

### Proline accumulation

In the initial experiment where only ANU 001 callus was used the proline concentrations increased with increasing salt concentration (Fig. 2.6). The increase became significant at the 200 mM level of salt and this was not different from the 400 mM.

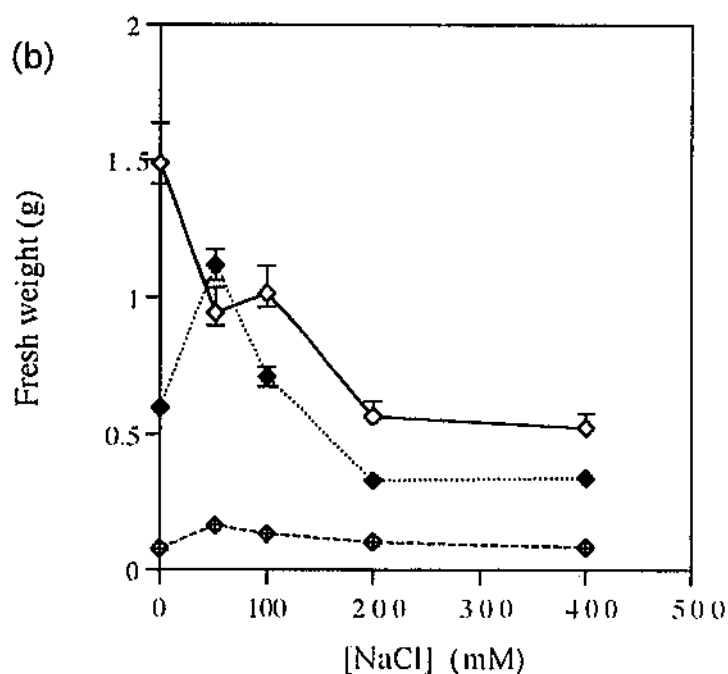
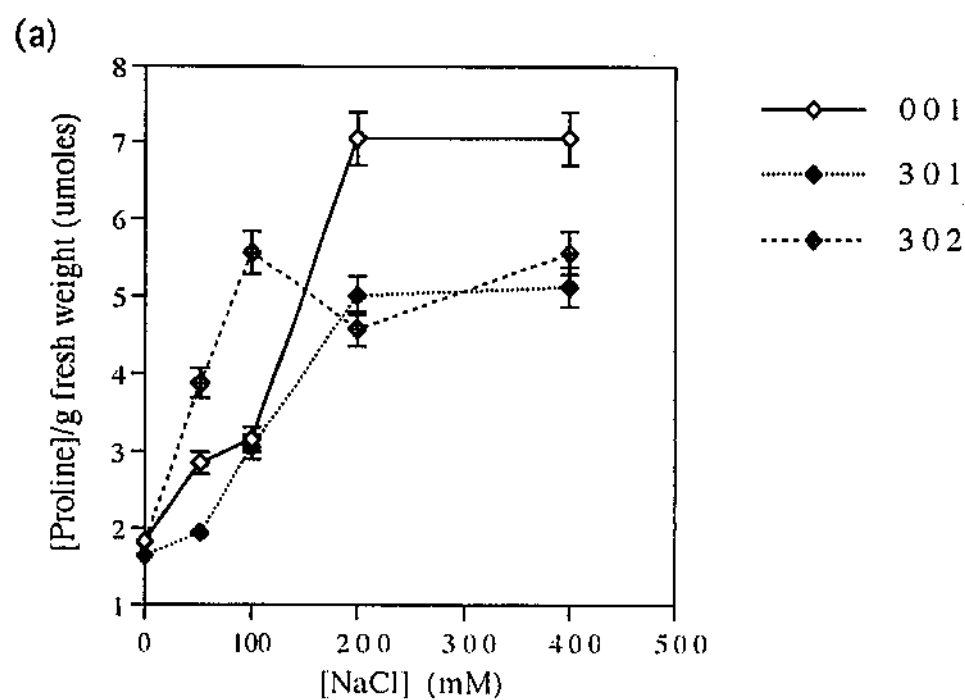
When three clones of *A. nummularia* were compared the proline concentration in all three clones generally increased as the level of salt increased. This increase was significant for clones ANU 001 and ANU 301 while there was no significant difference for ANU 302 (Fig 3.7). The comparisons of fresh weight, however, indicate that there is a difference between clones as well as an effect of treatment. Generally the fresh weight decreased with increasing salt concentration (Fig 3.7b).

**Fig. 3.6.** Mean proline concentration (umoles/g fresh weight) of *A. nummularia* callus (clone 001) grown in tissue culture at varying NaCl concentrations. Means were calculated from 5 replicates and vertical bars show standard error.





**Fig. 3.7.** Mean proline concentration (umoles/g fresh weight (a) and fresh weight (b) of callus cultures of three clones of *A. nummularia*. Means were calculated from 9 replicates and vertical bars show standard error.



The clones were not different in terms of the amount of proline produced at the different levels of salt, however ANU 001 and ANU 301 were affected by treatment. The multiple range test indicated ANU 001 and ANU 301 had equal amount of proline at 0 mM, 50 mM and 100 mM and increased significantly at 200 mM and did not change thereafter.

#### Fresh weight

The fresh weight of calli in all three clones generally decreased as the level of salt increased (Figure 2.7(b)). There was a significant difference between clones and all clones were affected by treatment. Using the multiple range test, ANU 001 showed highest fresh weight at 0 mM, while the other two clones showed highest fresh weight at 50 mM (ANU 301) or 50 and 100 mM (ANU 302).

### **3.4. Discussion**

The mechanisms that confer salt tolerance to cultured plant and plant cells are not completely understood. This experimental component of the project attempted to test the hypotheses put forward in chapter 1(1.3), in particular those relating to genetic variability between individuals of the same species at the tissue and cellular levels *in vitro*. Overall, it appears that there is a trend for proline accumulation as the level of salt increases and this varied between species. This is what could be expected given the information on proline accumulation in whole plants and how this varies between species.

For *A. nummularia* the clones differed in proline accumulation. ANU 001 accumulated more proline and had lower fresh weight than ANU 101. In this species the proline concentration and the fresh weight can be used to measure differences in salt tolerance between clones, however, it is difficult to determine at this stage whether the parameters measured are directly related to salt tolerance. For example, the percentage water content is higher in ANU 101 and this clone may not necessarily use proline for the adjustment of ionic balance. It is possible that it uses succulence as a means of coping with increased salt.

*A. stipitata* clones produced very low proline concentrations and the pattern of accumulation did not resemble that of the *A. nummularia* clones. Proline might not play an important role in salt tolerance in *A. stipitata*, other mechanisms of salt tolerance might be operating. One of the mechanisms might be increased succulence, as the water content is fairly high in both clones and is affected by the salt treatments. The lack of response to salt in the medium might be because the levels of salt were not high enough to induce a response from the shoots. If this is the case, it suggests *A. stipitata* may be more salt tolerant than *A. nummularia*.

*F. irregularis* showed a different pattern of proline accumulation and fresh weight. The proline level increased as the level of salt increased. The clone that accumulated less proline (FRAN 001) did not necessarily have higher fresh

weight. This result was different to that obtained for *A. nummularia*. It might be that proline is not the only mechanism that is used to tolerate salinity in *F. irregularis*. Succulence might also play a part in maintaining ionic balance.

A different trend of proline accumulation was encountered in callus cultures of the three clones of *A. nummularia*. Accumulation of proline increased gradually as the level of salt increased and leveled out at 200 mM. The clone that had higher proline accumulation did not, however, have the lowest fresh weight. Proline accumulation could not be used to differentiate between clones of *A. nummularia* callus.

Callus growth of *A. nummularia* was similar to that obtained for *A. undulata* observed by Smith and McComb (1981). Callus and shoot cultures of the same clone did not respond in the same way to salt (ANU 001). Callus decreased with increasing salt concentration while the shoots had an initial increase followed by a decrease in fresh weight, similar to that observed by Smith and McComb (1981) for whole plants. This may indicate that the growth of shoot cultures (whole organs) better reflects the type of response that the whole plant may produce. This suggests that salt tolerance is expressed differently at the cell and tissue level in *A. nummularia*. This supports the hypothesis of Smith and McComb (1981) that in halophytes, salt tolerance depends on the anatomical and physiological integrity of the whole plant, and cells of these plants have no ability to tolerate

NaCl once they undergo dedifferentiation to form callus. Given the variable responses in this work it would seem that further investigation needs to be done with other halophytic species to further develop this hypothesis.

The experiments described in the project have never been performed on these species. They need to be repeated under the same conditions in order to make more definite conclusions about the response of the species to salt in their environment. In doing this, there are a number of ways that the design of the experiments can be improved. Callus and shoot cultures of the same clones could be set up so that better comparisons could be made between the tissue and cellular levels. In shoot cultures growing at salt levels of 200 mM and 400 mM, shoots were sampled even if they were showing advanced chlorosis or had died. The sampling method could be improved so that the effects of chlorosis and death, if there are any, on proline accumulation could be eliminated. These experiments would help further determine whether proline accumulation and growth *in vitro* can be used to indicate the level of salt tolerance of shoot and callus cultures. This would also require further comparisons with the growth of whole plants.

## **4. GLASSHOUSE EXPERIMENTS**

### **4.1. Introduction**

The response to salt at the whole plant level has been reported in a number of halophytes. The growth of halophytes is stimulated by moderate levels of salt and inhibited by very high levels. This has been found in halophytes such as *A. spongiosa*, *Sueda monoica* (Storey and Wyn Jones, 1979) and *Salicornia bigelovii* (Ayala and O'Leary, 1995). Ashraf (1994) found that the salt tolerance of pigeon pea (*Cajanus cajan*) varied at three growth stages. It was also observed that in that particular species, increasing salt had an adverse effect on growth at all growth stages.

Glasshouse experiments were conducted to test the response of plants to varying salt concentrations at the whole plant level. The glasshouse environment provides factors that plants are likely to be exposed to when growing in their natural habitats. Screening is made easier in the glasshouse because root and shoot growth can be easily monitored. Other factors, both biotic and abiotic can also be controlled for the duration of the experiments. The following experiments were set up to examine the response of the species *A. nummularia* and *F. irregularis* towards salt at the whole plant level.

## **4.2. MATERIALS AND METHODS**

### **4.2.1. Salt treatment**

Cuttings of three clones of *A. nummularia* and *F. irregularis* were set up in pots containing 4 kg of steam pasteurised soil. For each species 20 pots were set up, each containing three clones. All pots were treated with nutrient solution (Thrive, 8g/10L) for a period of four weeks after which salt treatments commenced. Half of the pots were used for the salt treatment and the other half as controls. The required amount of salt was dissolved in full strength nutrient solution and the conductivity of the solution was measured. The different concentrations of NaCl used were 0 mM (control), 200 mM, 400 mM, 800 mM and 1000 mM. The salt concentration was increased in increments of 200 mM at two weeks intervals. The pots had a single drainage hole at the base and were flushed with the appropriate solutions. Flushing stopped when the conductivity of the incoming and the outgoing solutions were equal. Pots were watered to field capacity every two days.

### **4.2.2. Harvesting**

Fully expanded leaves from half of the control pots and the treated pots were sampled for proline two weeks after salt was increased. For the *A. nummularia* clones, 0.5 g of leaf tissue was used and 0.1 g of *F. irregularis*. The plants were tested for proline after growing at 0 mM, 200 mM, 400 mM, 600 mM, 800 mM and 1000 mM. Two weeks after the 1000 mM treatment had been reached the

plants that were not used for the proline test were harvested for fresh weight, dry weight and water content.

#### 4.2.3. Experiment 2.

A second experiment was set up in the glasshouse using only *F. irregularis* clones to determine optimum salt concentrations for growth. This was done because the control plants died in the first experiment. Twenty-four pots were set up each containing 4 Kg of steam pasteurised soil. The pots were treated with nutrient solution (8g/10L thrive) for four weeks and then treated with salt. The salt concentrations used were 0 mM (control), 50 mM, 100 mM, 200 mM, 400 mM and 800 mM. At each concentration there were four replicates and three clones. The NaCl concentrations were increased by 100 mM every day until the final concentrations were reached. Conductivity measurements were done as described above (3.2.2). The field capacity for each pot was determined and maintained with de-ionised water and plants were left to grow for four weeks. A fully expanded leaf was sampled from each plant from all treatments to determine proline concentration. All the plants were measured for fresh weight, dry weight and the percentage water content after they had been growing for five weeks at the different salt treatments.



### **4.3. Results**

#### **4.3.1 *A. mummularia***

All plants of all clones growing in salt showed slight chlorosis after being treated with 600 mM salt solution and salt crystals were found on the leaves of plants growing in the salt treatments. After being treated with 800mM salt, the leaves of the plants wilted, some plants did not recover. Chlorosis persisted in fully expanded basal leaves which eventually died and fell off the plants.

#### **Proline content**

The proline test was performed on both control plants and salt treated plants every two weeks. The proline concentration in control plants were higher than in salt treated plants until 800 mM in ANU 001 and 400 mM in 301 and 302 (Fig. 4.1). In ANU 001, the proline concentration decreased at 400 mM and then increased gradually. The other two clones showed gradual increase in proline as the salt concentration increased. Analysis of the results showed that in ANU 001, there was significant differences between the control plants and the salt treated plants growing at 400 mM and 1000 mM but no difference between control and salt treated plants at 600 mM and 800 mM. There were significant differences at all the salt levels between the control and the salt treated plants for ANU 301 and for ANU 302, there were differences in plants growing at control levels and 800 mM and 1000 mM (Fig. 4.1).

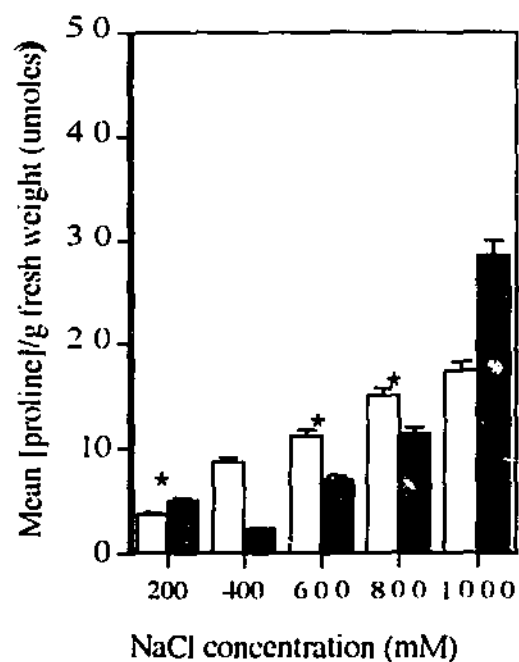
**Fig. 4.1.** Mean proline concentration (umoles/g fresh weight of the shoots of three clones of *Atriplex nummularia* grown in the glasshouse at varying NaCl concentrations. Means were calculated from a replication of 10 and vertical error bars show standard error.

□ - Salt  
■ + Salt

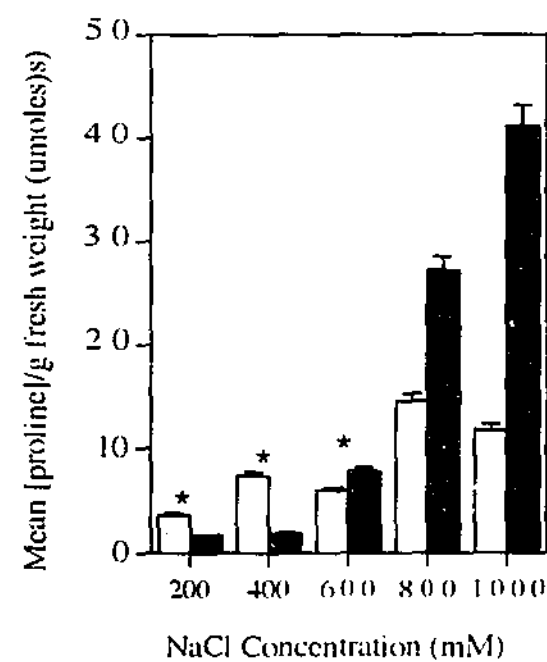
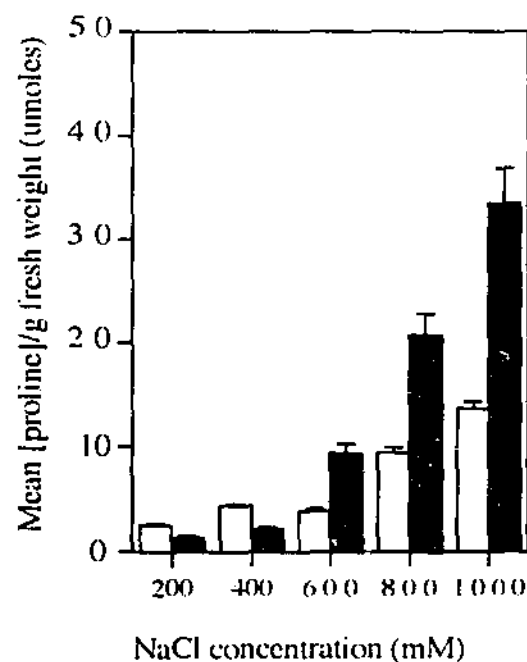
ANU 001

ANU 301

ANU 302



(\* results not significant)



### Productivity

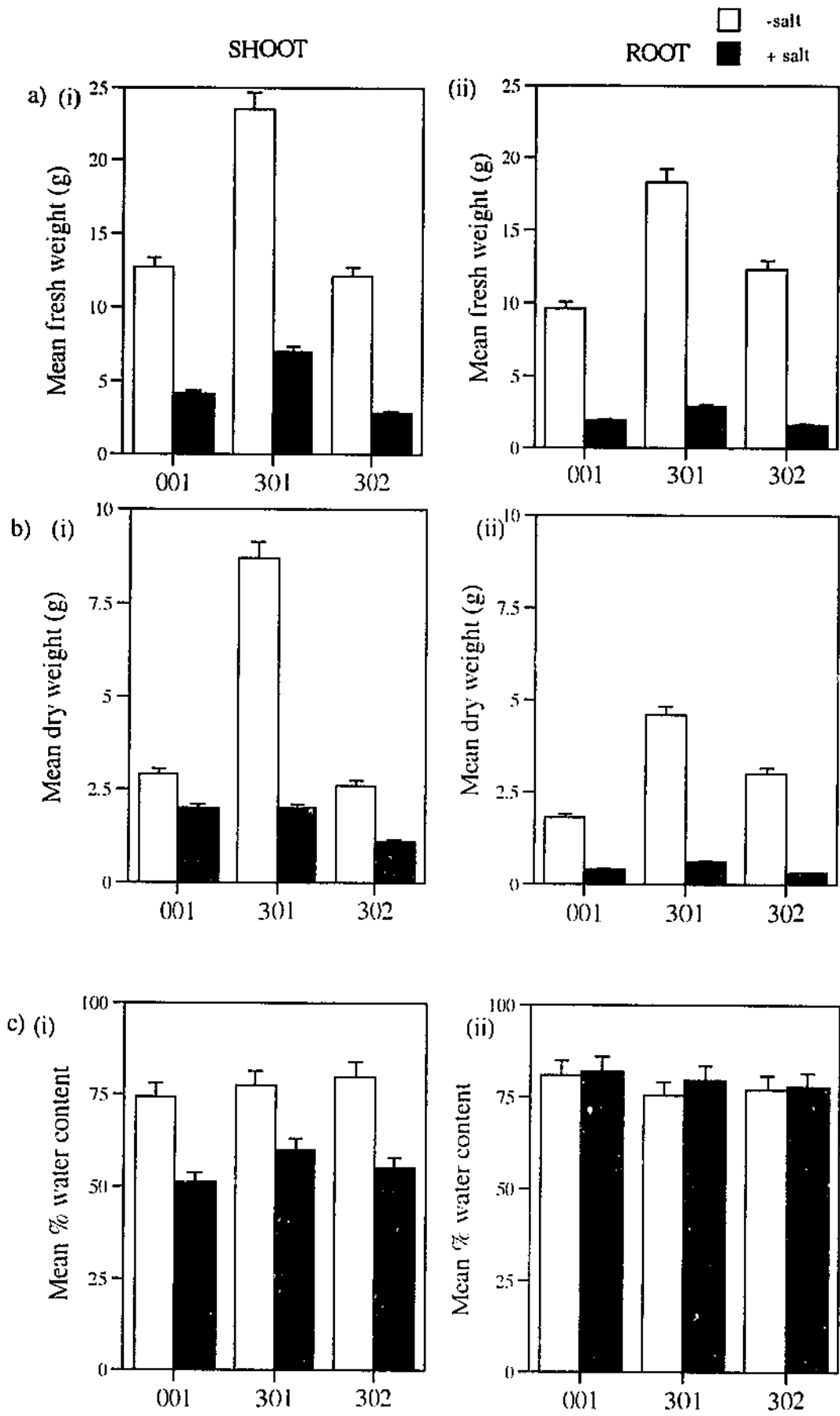
The clones were expected to show differences in productivity because they were genetically different. This is illustrated in figures 4.2a & b. The clones were found to be different in all measurements for both shoots and roots. The shoots and roots of the ANU 301 generally grew more than the other two clones. All clones showed significant differences between plants growing in salt and control plants, both in shoot and root growth. In root growth there was no treatment effect but the clones were found to be different. Clones were found to be equal in percentage water content and ANU 301 and ANU 302 showed treatment effects.

The percentage increase in fresh weight, dry weight and percentage water content of all plants were calculated compared to the control plants. The results obtained gave a better indication of which clones grew better when salt is introduced in their environment. Figure 4.3. illustrates that ANU 001 showed a higher percentage increase in fresh weight and dry weight. This result can be compared to the results obtained for the proline test, whereby ANU 001 showed higher proline production at higher salt levels than the other two clones.

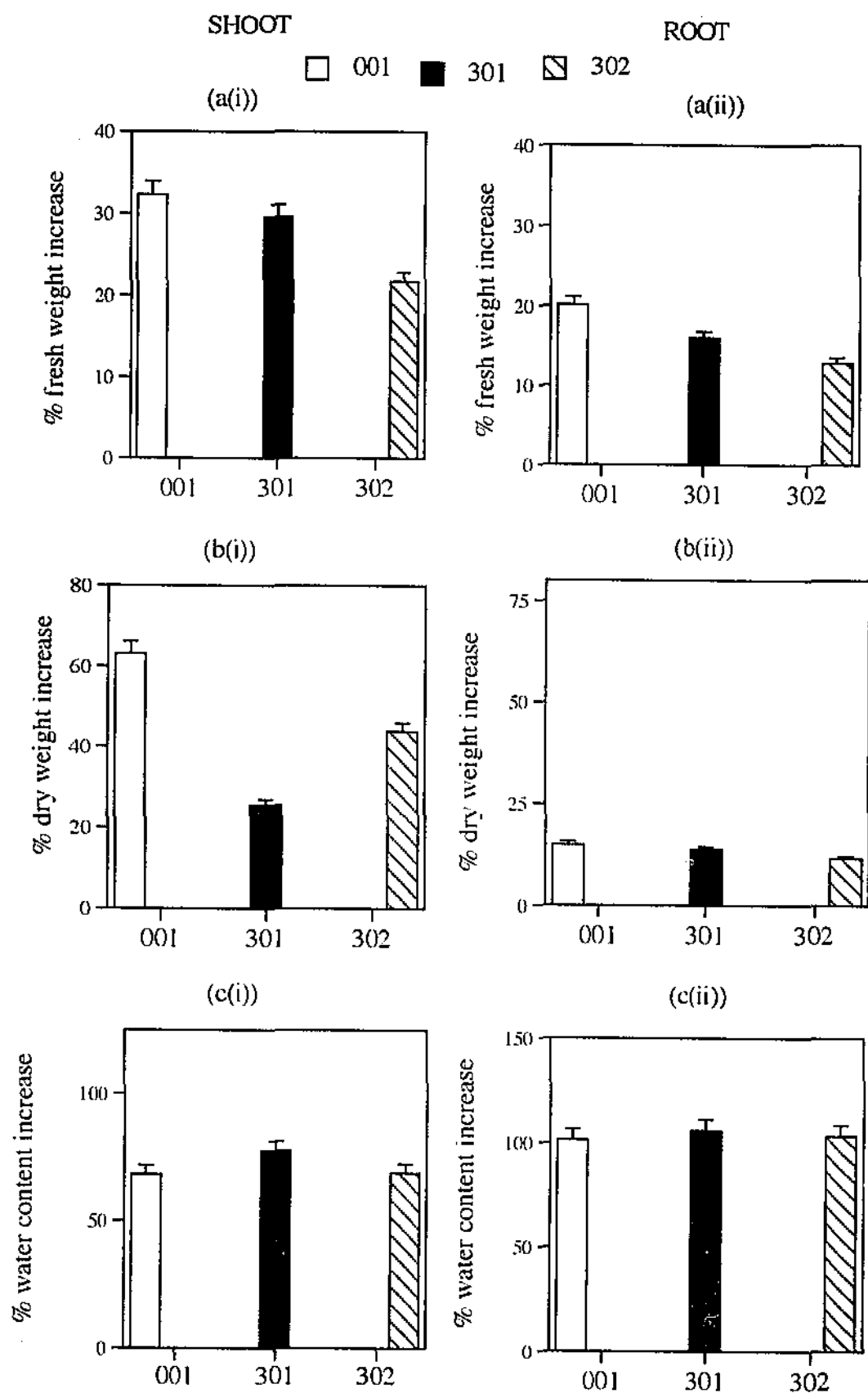
#### 4.3.2. *F. irregularis*

*Frankenia* clones did not grow as well as *Atriplex* clones at the different levels of salt. After four weeks all the plants at the control level had died. Plants treated with salt died after 800 mM salt was administered. Chlorosis of fully expanded

**Fig. 4.2.** Mean fresh weight (a), dry weight (b) and % water content (c) of the shoots (i) and roots (ii) of three clones of *A. nummularia* grown in the glasshouse. Means were calculated from a 10 replicates and vertical error bars show standard error.



**Fig. 4.3.** Relative increase in fresh weight (a), dry weight (b) and percentage water content (c) of three clones of *A. nummularia* shoots (i) and roots (ii) grown in 1000mM salt compared to control plants.



basal leaves occurred after 400 mM. As the plants died less plants were available for harvest for proline determination and therefore a second experiment was set up for the *Frankenia* clones.

### Experiment 1

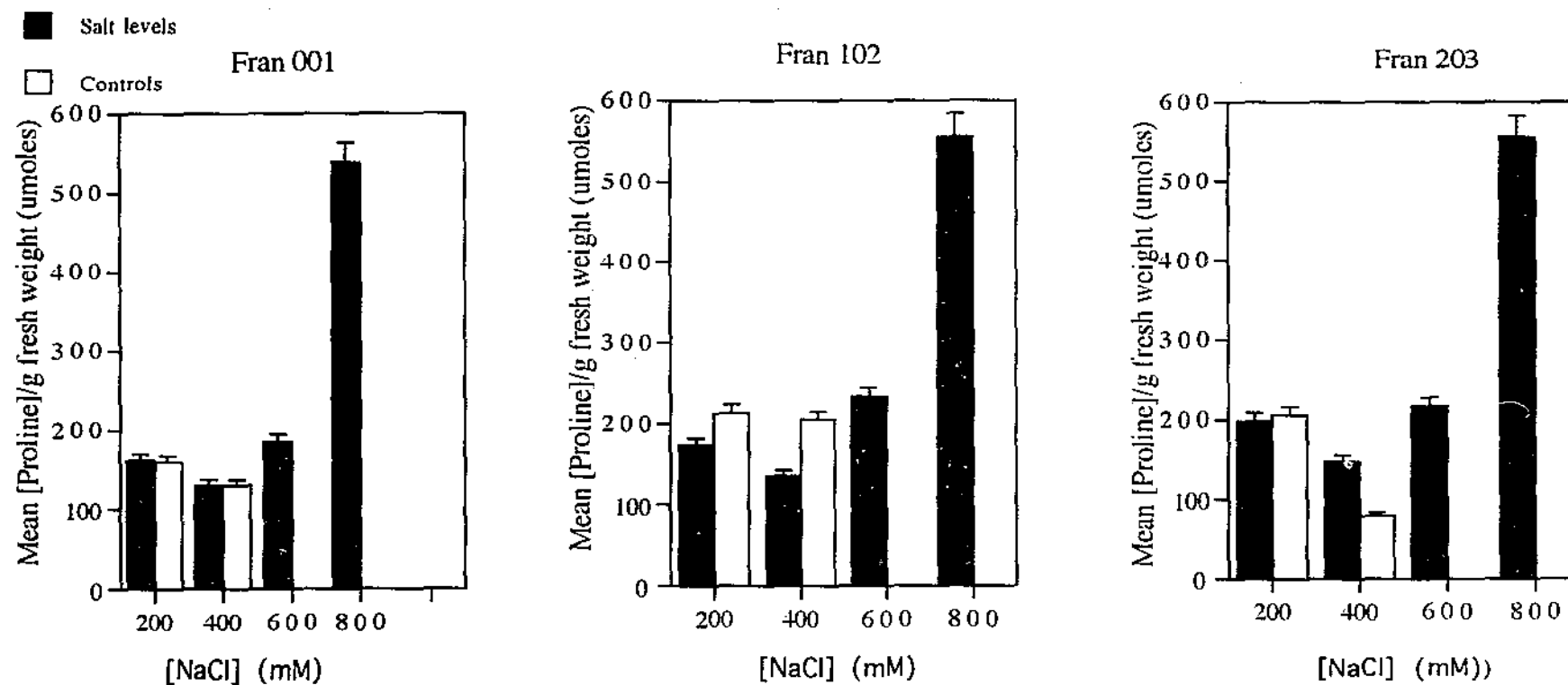
#### Proline content

The proline test was only performed on live plants and dead plants were not sampled. The proline concentration of salt treated plants decreased at 400 mM for all clones and then increased thereafter (Fig. 4.4). The clones were found to be equal in the amount of proline accumulated. The trend in proline concentration of salt treated plants compared to control plants cannot be analysed because of the death of the control plants.

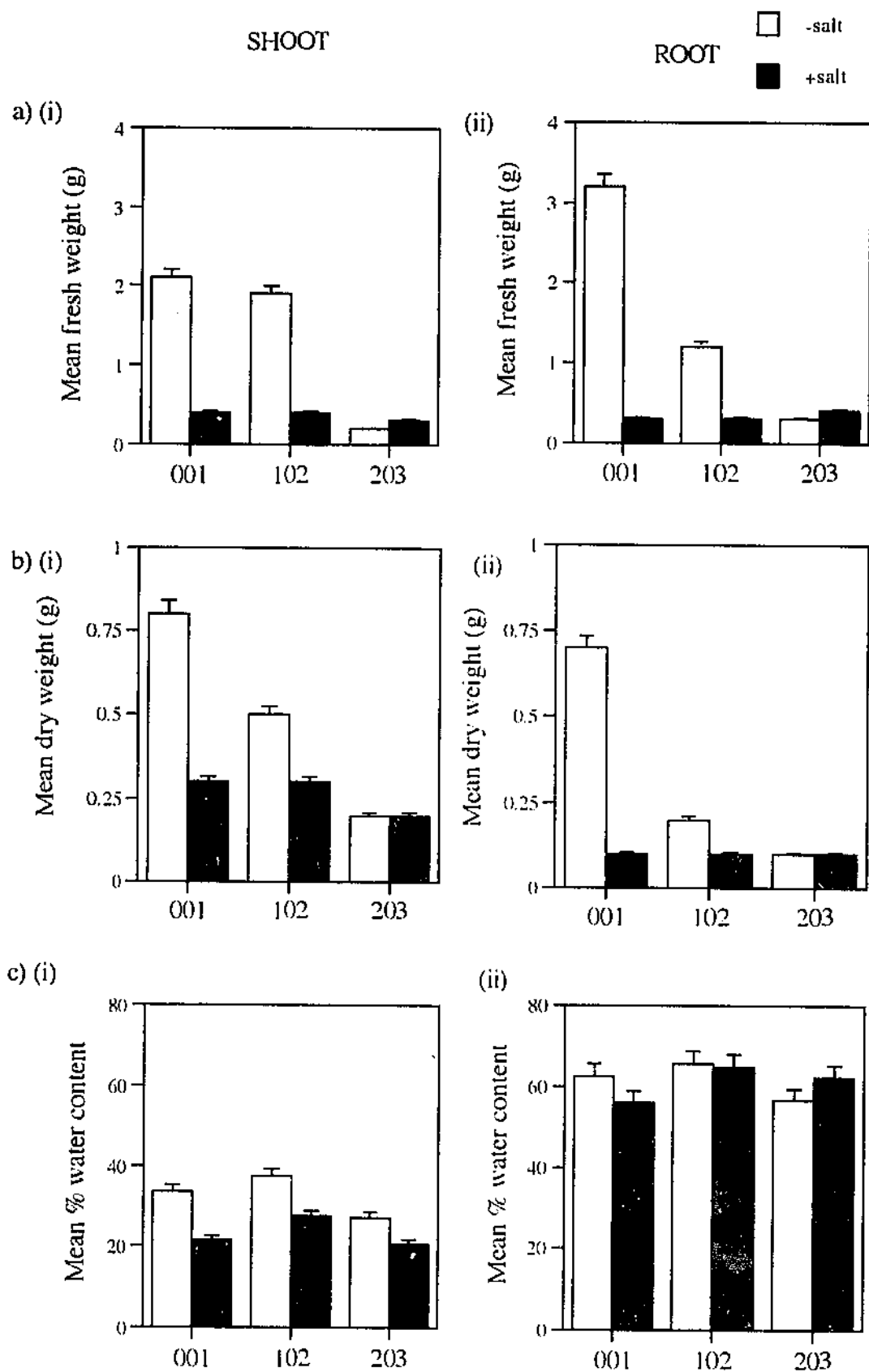
#### Productivity

At the time that plants were harvested, most were dead and all plants were used to measure productivity. The fresh weight measurements therefore do not give a good indication of productivity of the different clones. Analysis of the dry weight of shoots shows that there is no difference between clones. FRAN 203 showed a higher percentage growth increase in both shoot and root (Fig. 4.6). Unfortunately the results cannot be used to give a good indication of the true differences between clones.

**Fig. 4.4.** Mean proline concentration (umoles/g fresh weight of the shoot of three clones of *Frankenia irregularis* grown in the glasshouse at varying NaCl concentrations. Means were calculated from a replication of 10 and vertical bars show standard error.

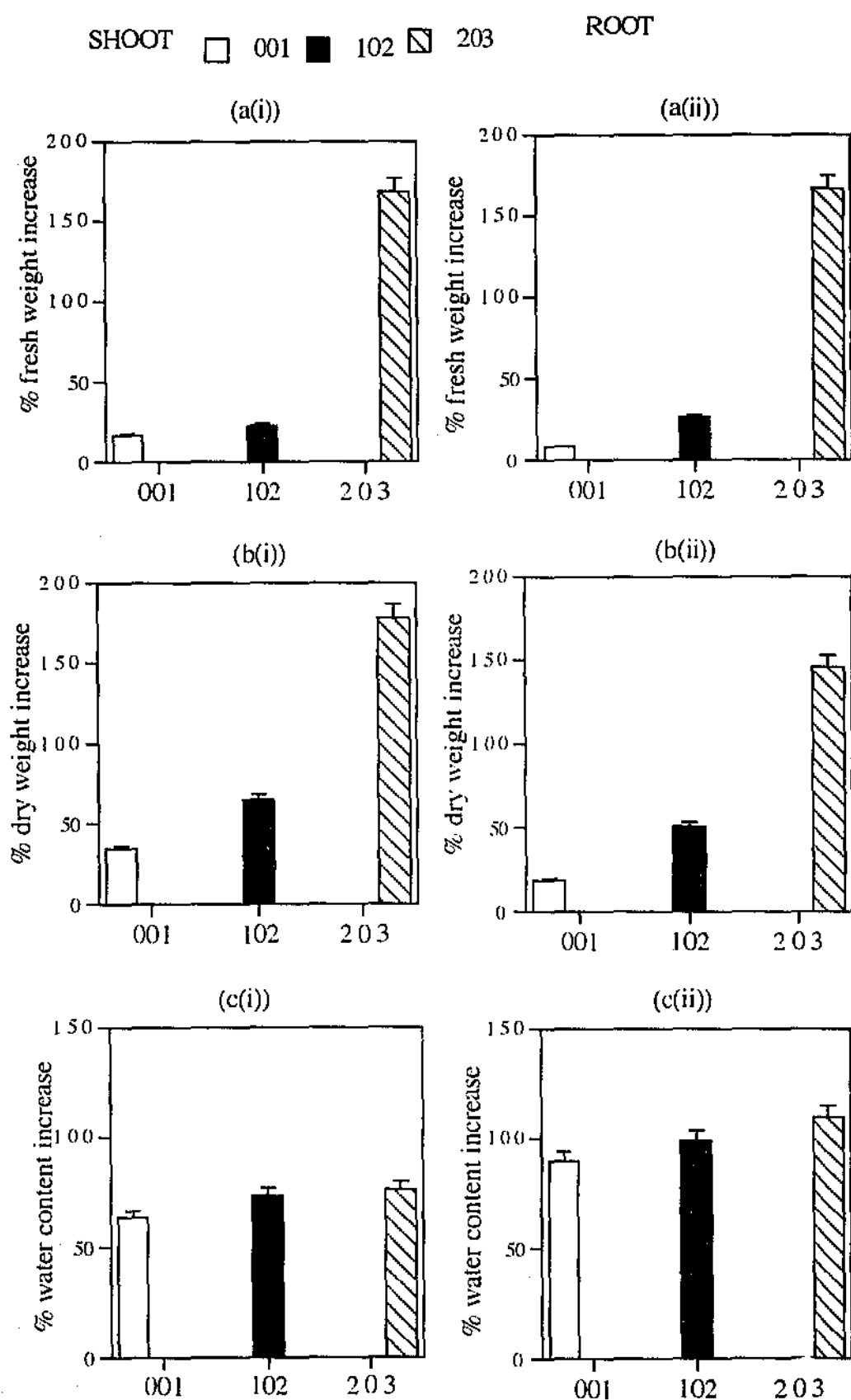


**Fig. 4.5.** Mean fresh weight (a), dry weight (b), and % water content (c) of the shoots (i) and roots (ii) of three clones of *F. irregularis* grown in the glasshouse. Means were calculated from 10 replicates and vertical error bars show standard error.

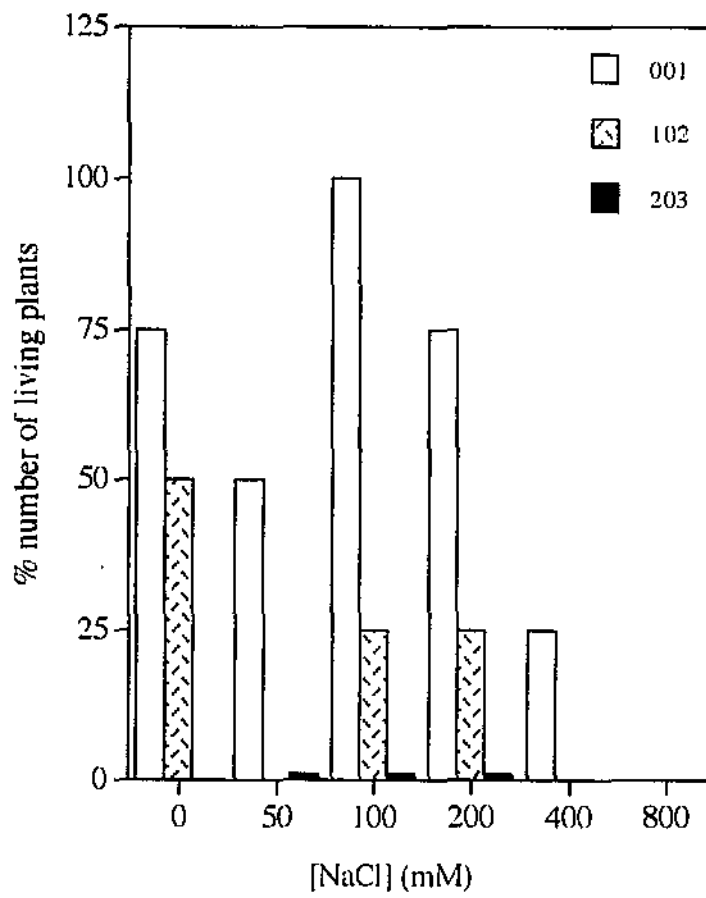




**Fig. 4.6.** Relative increase (expressed as %) in fresh weight (a), dry weight (b) and percentage water content of *F. irregularis* shoots (i) and roots (ii) grown at 1000mM salt compared to control plants.



**Fig. 4.7.** Percentage living plants of three clones of *Frankenia irregularis* after being treated with different levels of salt.



## Experiment 2

Experiment 2 was set up to find the response of *Frankenia* clones at the different levels of salt. All plants were harvested to measure proline. At 100 mM and 200 mM there were plants of all clones that were still alive, all plants died at 800mM. The clone FRAN 001 survived at all concentration except 800 mM and FRAN 203 showed the least percentage survival (Fig. 4.7).

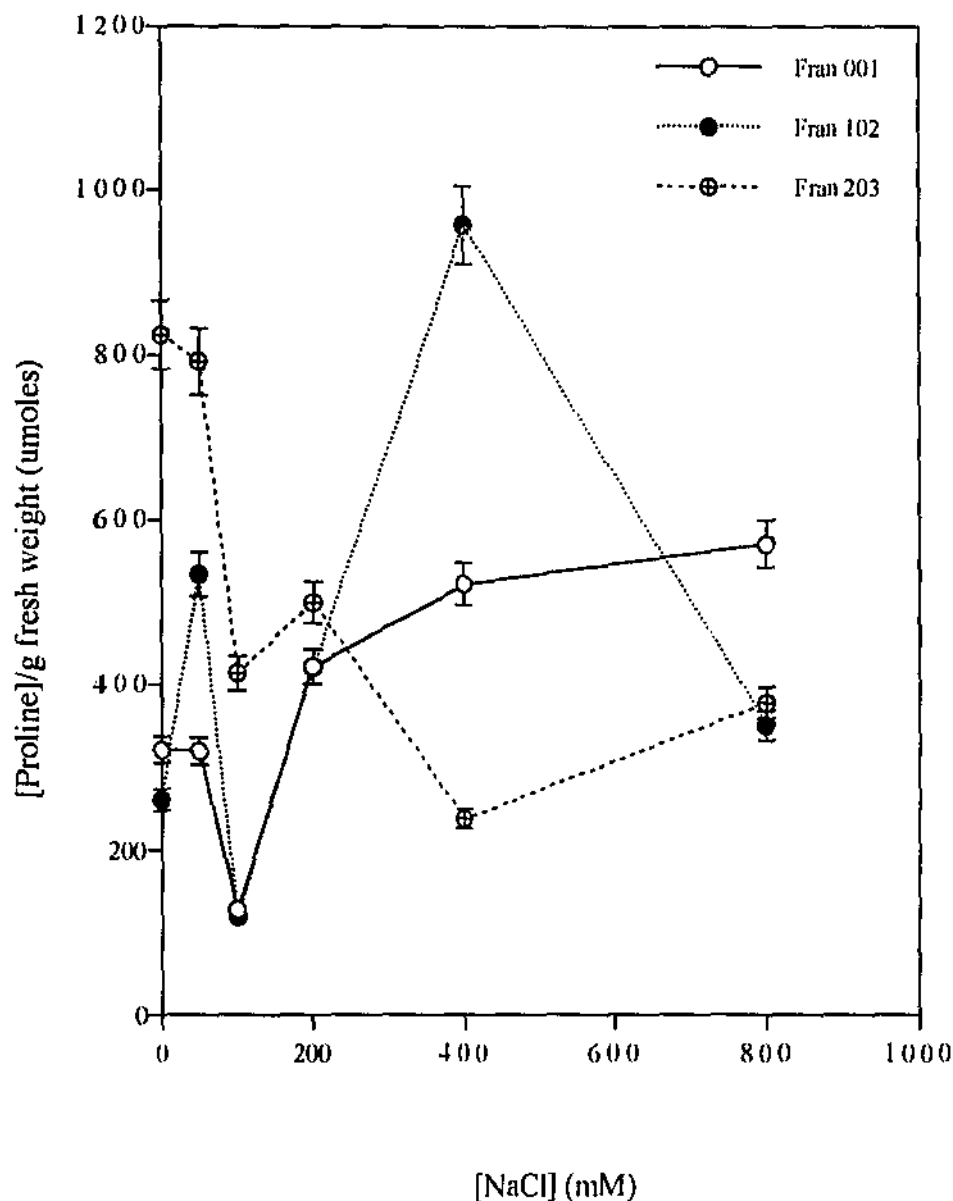
## Proline content

The proline concentration of all clones had a tendency to decrease at 100 mM salt and generally increased thereafter (Fig. 4.8). Clones were found to be equal in proline concentration and did not show any treatment effect. FRAN 001 showed the lowest concentration of proline at all levels of salt except 800 mM (Fig. 4.8). This was not statistically significant.

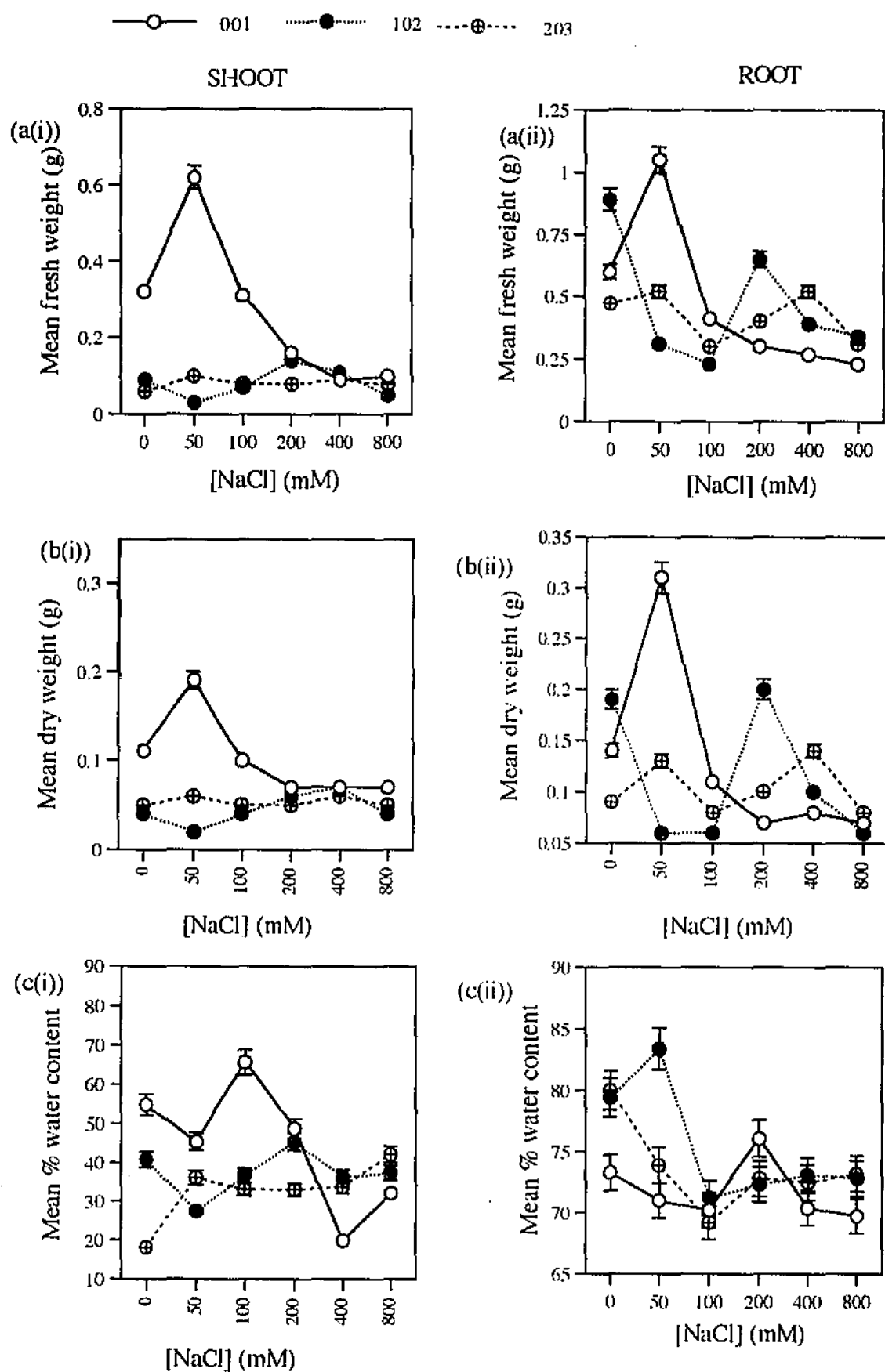
## Productivity.

As in experiment 1, the productivity measurements of the *Frankenia* clones could not be used to draw conclusions about the different clones especially at concentrations where most plants died. FRAN 001 might be expected to have higher fresh weight because it had the highest percentage of plants alive at all levels of salt. However, at 100 mM and 200 mM, where there were live plants of all clones, fresh weight and dry weight were equal for all clones. Clones were found to be significantly different for all shoot measurements.

**Fig. 4.8.** Mean proline concentration (umoles/g) fresh weight of *F. irregularis* clones grown in the glasshouse at varying NaCl concentrations. Means were calculated from a replicate of 4 and vertical bars show standard error.



**Fig. 4.9.** Mean fresh weight (a), dry weight (b) and % water content (c) of the roots and shoots of three clones of *F. irregularis* grown in the glasshouse at varying NaCl concentrations. Means were calculated from 4 replicates and vertical error bars show standard error.



### 4.3. Discussion

Clones of *A. nummularia* and *F. irregularis* showed variable responses to salt at the whole plant level. Both species responded by excreting salt from the leaves.

*A. nummularia* showed low accumulation of proline and the pattern of proline production observed was similar to that obtained by Storey and Wyn Jones (1979) in their work with *A. spongiosa* and *Suaeda monoica*. The proline accumulation was high in plants grown at high salinity as well as in control plants. It appears that the tissues were stressed at very low and very high levels of salt. A variety of compounds have been suggested as having a function in osmotic adjustment including carbohydrates, organic acids and sugar alcohols (Stewart and Lee, 1974). It is possible that in *A. nummularia*, other compounds also take part in osmotic adjustments.

The water content of the shoots (Fig. 4.2c(i)) suggests that *A. nummularia* might use succulence to cope with salinity. Another measure of succulence perhaps that used by Osmond *et al.* (1989) is needed to confirm this.

It cannot be concluded that at low proline concentrations the fresh weight is high in *A. nummularia* clones. The fresh weight is not a good measurement of growth in this species because it has a high percentage water content. The

design of the experiment could be altered by growing plants at different salt levels, as was done in experiment two with *Frankenia* (4.3.2). This would allow the level of salt at which there is optimum growth to be determined. If the replication number was higher the role of succulence in terms of percentage water content could be more obvious. These alterations would allow the duration of the experiment to be extended and the use of dry weight, a measurement of productivity that would be more useful.

*F. irregularis* clones accumulated high levels of proline and died at control levels and at 600-800 mM salt. Proline might be the major osmoprotector in *F. irregularis* and its overproduction at high salinity could have caused an osmotic imbalance and resulted in the death of the plants. As in tissue culture dead as well as live plants were sampled. The effect of that on the experimental results is not known.

It cannot be said that *Frankenia* does not use succulence for osmotic adjustments although the water content of the *Frankenia* clones were quite low. This can be determined if the replication number of experiment two is increased and only live plants are used for productivity measurements.

The salt tolerance of the two species could be due to different mechanisms and the amount of proline accumulated could be due to the differences in the relative volumes of the cytoplasm and the vacuole. If the above assumptions are true, the role of proline in osmotic adjustment cannot be common for all halophytes. The

fact that *F. irregulars* clones did not grow as well and accumulated more proline than *A. nummularia*, suggests that the species might have different levels of adaptations to salt. Proline accumulation and relative productivity can be used to differentiate the level of salt tolerance between genotypes within a species but direct comparisons between species are probably not possible.



## 5. GENERAL DISCUSSION

The results of the project indicates further that salt tolerance is a complex response. The project attempted to find out if proline accumulation and productivity can be used to indicate the level of salt tolerance.

The different species used in the project, have different ways of tolerating salt. *A. nummularia* for instance does not seem to use proline as a major osmoprotectant at the whole plant level, tissue level and cellular level. *F. irregularis* seems to use proline as an osmoprotectant at the whole plant level and at the tissue level and there seems to be other processes operating at the tissue level.

In all the species studied, fresh weight was not the best way to measure productivity at the whole plant level because of the death of plants but it may be reasonable indicator of succulence. Productivity measurements would be improved by designing experiments so that fresh weight and dry weight were measured at each sampling period. This was not feasible because the replication required to do this was impractical.

All the species studied were found to have high percentage water content with the higher salt concentrations. This gives a good indication that succulence might be used to maintain turgor. However, another measurement of succulence should be used to support this. Osmond *et al.* (1989) used a ratio of water content and chlorophyll content in their measurement of succulence for *Agave deserti*. This

method appears to be more reliable but also requires more plant material (more replicates) and time.

The responses of whole plants, tissues and cells can be better investigated if the same clones were used at all levels. The restrictions that prevented this being done in the project were mainly due to the limitation of time, the unavailability of plant material, including the difficulty of establishing new callus cultures. There are, however, indications that the tolerance to salt is different at these levels for some of the species tested.

*A. nummularia* and *F. irregularis* clones showed measurable differences in their response to salt treatments. *A. stipitata* clones showed no significant responses to salt in all parameters measured. It may be that the salt levels were not high enough to obtain a response.

The death of *F. irregularis* plants in experiment 1 (4.3.2) and higher proline levels of *A. nummularia* (Fig. 4.1) at the control level suggests that the control plants may have been stressed. The percentage relative increase in growth of control plants, therefore, was not necessarily an effective measure of salt tolerance. Dry weight rather than fresh weight could be considered as a more effective productivity measurement because of the high water content of the species. There was a limitation on which parameter to use because of the death of plants in the glasshouse.

There are indications that there is a genetic difference in the accumulation of proline in response to salt in two of the species tested. Further investigation in proline accumulation and growth is needed to determine whether high proline or low proline is an indicator of salt tolerance. Evidence suggests that proline may be merely an indication of stress rather than an adaptation for salt tolerance. In which case low proline at high salt levels may be an indication of higher salt tolerance.

Proline concentrations can be used to measure genetic variability within a species. This is evident at the tissue level. The results obtained suggests that at the cellular and whole plant levels, proline concentration cannot be used to differentiate between individuals of the species studied. Further investigations and alternative experimental designs need to be carried out to obtain more conclusive results.

Further studies are required that correlate proline production with other parameters (eg. relative productivity or survival at different salt concentrations) before proline can be used as a measure of salt tolerance. In addition, it appears that the role of proline varies between species and for some species it may be a more useful measure than for others.

The use of proline as an indicator for salt tolerance could be developed with further investigations using salt tolerant plants. Proline can, in some cases, be used to distinguish between genotypes within a species. However, more studies on the production of proline, and the mechanisms involved, are required before its production can be used to assist in breeding of salt tolerant plants.

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